The role of Fyn in regulating T cell responses

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A thesis presented for the degree of Doctor of Philosophy in the University of London, 2005

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Acknowledgments

Firstly I would like to thank my supervisor Dr Rose Zamoyska for providing me with the opportunity to undertake a PhD and, most importantly, for her continual help and guidance throughout these 3 years. I would especially like to thank Dr Matthew Lovatt and Dr Ben Seddon, who have helped me immensely with the technical and theoretical aspects of the project. Also thanks to Dr Lisa Miosge for patiently proofreading this thesis, Miss Ihjaaz Qureshi for showing me how to conduct killing assays, Mr Peter Tomlinson for preparing GI tracts from mice and also conducting ELISAs, and Miss Meenaxi Chavda for genotyping the mice.

Outside of the Zamoyska lab I would like to thank Dr Marc Veldhoen for setting up and running all the real-time PCR experiments presented in this thesis, this was an invaluable contribution to the project. Also I would like to thank all members of the Molecular Immunology and Immune Cell Biology divisions for their input to this thesis, especially Dr George Kassiotis for helpful discussion and Dr Enrique Veiga-Fernandez and Dr Mark Coles for help with microscopy.

The final and most significant thank you goes to my wife Farnaz who has been my support over the last few months; I really do not think I could have done it without her love and patience.

Abstract

We have addressed the role of Fyn in T cells using two different transgenic mouse systems. Using the class I-restricted F5 TCR transgenic model, we found that Fyn$^{-/-}$ CD8 cells showed normal triggering and entry into cell division over a range of peptide concentrations, but subsequently proliferated more than WT CD8 cells. Increased proliferation of F5 Fyn$^{-/-}$ cells was driven by their ability to produce more IL-2, which occurred only after cross-linking TCR and CD8 molecules with antibodies or pMHC, not by triggering through the TCR alone. Additionally, F5 Fyn$^{-/-}$ cells showed differences in the induction of specific AP-1 members that could influence IL-2 production. F5 Fyn$^{-/-}$ cells required only 1 hr stimulation with Ag to commit to IL-2 production, whereas F5 WT cells needed ~5 hrs, and yet IL-2 production by Fyn$^{-/-}$ cells remained sensitive to the Src-inhibitor PP2, added after 3 hrs. These data suggest that loss of Fyn uncouples Lck activity from the need for sustained exposure to antigen and therefore, that Fyn acts as a negative regulator to limit IL-2 production in CD8 T cells. Not only was proliferation of F5 Fyn$^{-/-}$ cells increased compared to WT controls, but so was the CTL response as judged by effector molecule expression and also specific killing activity. Like the increase in proliferation, the improved generation/function of Fyn$^{-/-}$ CTL compared to WT was dependent on the levels of IL-2 produced by these cells.

In our second transgenic model, Fyn$^{-/-}$ mice expressing reduced amounts of Lck were generated. These animals develop a fatal lymphoproliferative disorder, characterized

by gut pathology and weight loss, an expansion of effector-memory like $CD4^+CD44^+$ cells, and the appearance of anti-nuclear antibodies (ANA). Disease was ameliorated by turning off Lck expression, suggesting that an appropriate balance of Lck and Fyn activity is required *in vivo* to achieve cell survival without stimulating autoimmunity. Our data provides functional *in vivo* and *in vitro* evidence for a regulatory role for Fyn in both CD4 and CD8 T cells.

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Abbreviations

%	Percentage
~	Approximatly
-/-	Homozygous knockout
ADAP	Adhesion and degranulation promoting adapter protein
Ag	Antigen
AICD	Activation-induced cell death
ANA	Anti-nuclear antibodies
APC	Antigen preseting cell
Apc	Allophycocyanin
APS	Ammonium persulphate
BCR	B-cell receptor
Bio	Biotinylated
BMDC	Bonemarrow-derived dendrtitic cell
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CD28RE	CD28 response element
CD	Cluster of differentiation
CFSE	5(6)-Carboxyfluorescin diacetate, succinimdyl ester

CLIP	Class II-assocaited invariant-chain peptide
CO_2	Carbon dioxide
CRAC	Cytoplasmic Ca^{2+} release activated Ca^{2+} channels
cSMAC	Central supra-molecular activation cluster
C-termini	Carboxy-termini
CTLA-4	Cytolytic T-lymphocyte associated antigen 4
CTL	Cytotoxic lymphocyte
DAG	Diacyl glycerol
DC	Dendritic cell
dH_20	De-ionised water
DMSO	Dimethyl sulphoxide
DN	Double negative thymocyte
DNA	Deoxyribonucleic acid
DOX	Doxycycline
DP	Double positive thymocyte
EAE	Experimental autoimmune encephalomyelitis
ECL	Enhanced chemi-luminescence
ELISA	Enzyme-linked immunosorbent assay
ERK	Extra-cellular signal related kinase

FACS	Fluorescence activated cell sorter
FITC	Fluorescein isothyiocyanate
FRK	Fos-regulatory kinase
FTOC	Foetal thymic organ culture
GEF	Guanine nucleotide exchange factor
GEM	Glycolipid-enriched microdomain
GI tract	Gastro-intestinal tract
HAART	Highly active ant-retroviral therapy
HI-FCS	Heat-inactivated foetal calf serum
HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
Hrs	Hours
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
IMDM	Iscove's modified dulbecco's medium
IP_3	Inositol-1,4,5-triphosphate
ITAM	Immunoregulatory tyrosine activation motif
ITIM	Immunoregulatory tyrosine inhibitory motif
i.v	intravenous

J	Joining segment
JAK	Janus-activated kinase
JNK	c-Jun N-terminal kinas
kB	Kilo-bases
kDa	Kilo-daltons
LAT	Linker of activated T cells
LCK	Lymphocyte restricted kinase
LCR	Locus control region
LFA-1	Leukocyte functional antigen
LN	Lymph node
LPS	Lipo-polysaccharide
M	Molar
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MKK	Mitogen activated kinase kinase
MLN	Mesenteric lymph node
mM	Milli-molar
μM	Micro-molar

NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NK	Natural killer
NO	Nitric oxide
N-termini	Amino-termini
PAG	Phosphoprotein associated with GEMS
PAMP	Pathogen-assocaited molecular patterns
PBL	Peripheral blood leukocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent protein kinase 1
PE	Phycoerythrin
PEP	Proline-glutamicacid-serine-threonine-rich domain phosphatase
PIP_2	Phosphatidyl inositol-4,5, bisphosphate
PI-3K	Phosphoinositol-3 kinase
PKB	Protein kinase B
PKC	Proetin kinase C
PLC	Phospholipase C
PLN	Peripheral lymph node
pMHC	Peptide-loaded major histocompatability complex
PRR	Pattern recognition receptor

pSMAC	Peripheral supra-molecular activation complex
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTK	Protein tyrosine kinase
RAG	Recombinase activating gene
RNA	Ribonucleic acid
RT	Room temperature
SCID	Severe combined immunodeficiency disorder
SDS-PAGE	Sodium Dodecyl sulphate polyacrylamide gel electrophoresis
SEA	Staphylococcal enterotoxin A
SH-	Src homology domain
SLAM	Signal lymphocyte activation molecule
SLP-76	SH-2 domain-containing leukocyte protein of 76kD
SOS	Son of sevenless
SP	Single positive thymocyte
SPI	Small protease inhibitors
STI	Structured antiviral therapy interruption
TCR	T cell receptor
T_H	T helper cell
TLR	Toll-like receptor
T_{reg}	T regulatory cell

V	Variable segment
Vβ/α	Variable region of TCR α or β chains
WASp	Wiscott Aldrich syndrome protein
ZAP-70	Zeta-associated protein of 70 kDa

Chapter 1: Introduction

1.1 The branches of the immune system

Our immune system wages a constant battle against infectious agents present in the environment and is composed of several cell types that eliminate bacteria, viruses and other foreign agents from our bodies. This response can generally be subdivided into two branches called the innate and adaptive immune systems. Successful immunity from an invading pathogen is often achieved by the independent and synergistic functions of both these two systems.

1.1.1 The innate immune system

Innate immunity is considered to be the first line of defence against an invading pathogen. It functions by recognising the conserved features that are generally referred to as pathogen-associated molecular patterns (PAMPS), which are components of bacteria, fungi, protozoa and viruses. Due to its generalised nature, innate immunity can respond almost instantaneously to challenge and does not require prior exposure to the invading organism.

1.1.2 Protective barriers

The epithelium is the first component of the innate system that an infectious agent must overcome to gain a foothold in the body. Pathogens often cross the epithelium at internal sites such as the lungs or gastrointestinal (GI) tract as the outer skin is

relatively tough and impermeable. At the inner epithelial sites, a number of generalised mechanisms exist to prevent pathogen access. These include the secretion of mucus that can coat microorganisms preventing them from crossing the epithelial barrier, and chemicals such as lysozyme, present in tears and saliva, that inhibit microbial growth. Furthermore, epithelial cells are also bathed in a fluid containing anti-microbial agents such as surfactant proteins A and D. These bind pathogens and facilitate their removal by specialised cell types such as macrophages.

1.1.3 Macrophages and neutrophils

Macrophages are mononuclear cells that are present in tissues including the lung and GI tract. In contrast, neutrophils normally reside in the blood and migrate to the site of infection in response to soluble factors. Both cell types act by recognising and ingesting pathogens by virtue of receptors expressed on their cell surface. Phagocytosis is an active cellular process that involves the enclosure of the pathogen within the phagocytic membrane to form a structure called a phagosome. This then becomes fused with the cellular destructive machinery termed lysosomes, forming a phagolysosome. These structures contain enzymes such as lysozyme that mediates destruction of the pathogen. Macrophages and neutrophils also directly mediate the destruction of invading microorganism by the secretion of toxic molecules such as H_2O_2 and NO. Furthermore, macrophages can also secrete cytokines such as IL-1, IL-6, IL-12, and TNFα, and chemokines such as IL-8. The cytokines serve to activate other cells involved in the immune response such as T cells and DCs. However chemokines act by bringing other cells types including neutrophils and more

macrophages to the site of immune challenge. This massive tissue infiltration leads to localised inflammation. Moreover the macrophage-derived TNFα increases vascular permeability allowing access of blood born immune cells to the site of infection. Furthermore, TNFα, IL-1 and IL-6 can also raise body temperature slowing the rate of pathogen growth, as well as induce the production of acute-phase proteins that have properties similar to antibodies without the specificity. It should also be noted that activated macrophages upregulate the expression of specific surface molecules that enable them to act as professional APC and present antigen to T cells. Therefore, macrophages can also greatly influence the adaptive immune response.

1.1.4 Natural killer (NK) cells

NK cells are bone marrow derived cells that contain lytic granules much like the cytotoxic T cells of the adaptive immune system, however they are able to eliminate cells without prior antigen exposure. They are activated by macrophage-derived cytokines such as IL-12, and also by IFN secreted by virally infected cells. NK cells are also able to produce IFNγ that can help to suppress viral infection. Therefore, it is considered that NK cells act to suppress a viral infection for the period of time required to mount an adaptive immune response. It is thought that NK cells are able to distinguish self from non-self by virtue of the receptors they express. An example of an NK inhibitory receptor is Ly49 that will be able to recognise normal self-MHC class I molecules and prevent killing from occurring. If however viral infection has modified the MHC class I on the cell surface then these inhibitory receptors fail recognise the MHC as self and fail to prevent NK mediated lysis. In the mouse there

are also Ly49 NK receptors that recognise non-self MHC and have a pro-lytic effect. Therefore NK activity relies on an intricate balance of positive and negative signals. Furthermore, tumour cells often escape immune detection by CTL as they have lost MHC class I expression, however this makes them more susceptible to NK mediated lysis as there is no self-MHC molecule to prevent NK activation.

1.1.5 Mast cells

Mast cells are also components of the innate immune system and are found near surfaces in the body that are in contact with the environment, such as the GI tract, skin and airways. They express a number of receptors that enable them to respond to the presence of a pathogen, one of the most prominent being the high affinity IgE binding FCεRI receptor. Specific IgE produced by B cells during a Th2 immune response binds to the FCεRI receptor on the Mast cell. When the bound IgE encounters and binds its specific antigen this triggers the release of preformed granules containing pro-inflammatory mediators such as histamine (Galli *et al.*, 2005).

1.1.6 The complement system

One way in which foreign microbial agents are eliminated is through the complement system that is, in part, coupled to the expression of complement receptors on the surface of innate immune cells. In general the complement system is composed of multiple proteins that can opsonise pathogens for phagocytosis, activate inflammatory responses and also act directly on the invading organisms as proteases. In a resting

cell, the complement proteins are present in an inactive state and become activated through proteolyitic cleavage. There are three pathways that can activate complement. Firstly, binding of the C1q complement protein to the pathogen surface activates the so-called classical pathway. Secondly, when the mannan-binding lectin serum protein binds to mannose-containing carbohydrates on the pathogen surface, the mannan-binding lectin pathway can be triggered. Lastly, the alternative pathway of complement activation occurs when spontaneously activated complement components bind to the pathogen surface. All pathways then activate the C3 convertase protein that also binds to the pathogen surface and cleaves the C3 complement protein to form C3a, a pro-inflammatory mediator, and C3b that induces opsonisation. C3b-mediated opsonisation is regulated by the presence of complement receptors on the surface of macrophages and also mast cells, these include CR1 and CR3. Furthermore C3b also induces the formation of membrane attack complexes that create pores in the cell membrane of the pathogen, inducing death.

1.1.7 Pattern recognition receptors (PRR)

Although generally thought to recognise general pathogenic structures or compounds present early in an infection, the innate immune system does possess some elements of target specificity. An example of a PRR expressed by phagocytic cells is the macrophage mannose receptor that can bind specific sugar residues found on the surface of viruses and bacteria, aiding recognition. Moreover, phagocytic cells also express scavenger receptors able to recognise anionic polymers and acetylated low-density lipoproteins that would normally be shielded from recognition by the

presence of sialic acid modification on host derived cells. A further class of surface molecules important to innate immunity are the Toll-like receptors (TLR) that recognise the presence of various microbial constituents. For example TLR-4, in concert with macrophage receptor CD14, has been shown to signal the presence of LPS by switching on the expression of cytokine genes that include TNFα. Furthermore, TLR-2 has been shown to signal the presence of proteoglycans derived from gram-positive bacteria. These observations indicate that different TLR have different specificities for various pathogen products. Interestingly, TLR signalling may also assist the adaptive immune system by induces the expression of co-stimulatory molecules on APC that are required for T cell activation.

1.2 Adaptive immunity

While in most cases the innate immune response will be sufficient to prevent an infection from establishing a foothold, there are certain intrinsic failings associated with this response. Firstly, pathogens are constantly evolving and thus may escape the surveillance mechanisms of the innate immune systems due to major alterations in the PAMP. Secondly, some pathogens have natural mechanisms to evade recognition by the innate immune system. For example certain bacterial strains can secrete a thick polysaccharide that prevents recognition by PRR. Adaptive immunity overcomes many of the problems associated with the limited diversity of innate immunity in that it relies on the random generation of antigen receptor repertoires that are able to recognise almost any pathogen-derived peptide. The adaptive

immune response requires more time than the innate as it requires a given clone that is initially present at low frequencies, to encounter specific antigen and undergo clonal expansion and differentiate into an effector cell. Therefore it can take anywhere between 4-7 days until the adaptive immune system is able to mount an effective primary response against a pathogen. The adaptive immune system confers immunological memory, as upon a secondary encounter with the same antigen, the response is much more rapid due to elevated numbers of specific clones generated by the primary encounter and their pre-differentiated phenotype. In contrast, the innate immune response will be the same regardless of the number of times antigen is encountered. Therefore, it is clear that the initial response against a pathogen is mediated by the innate system, however even if this proves insufficient the delay allows for the preparation of an adaptive response. Moreover, several cells and cell products of the innate immune system have been shown to influence the function of adaptive immune cells, helping to improve pathogen clearance.

1.2.1 The generation of lymphocyte immune receptor diversity

B cells are generated in the bone marrow, whereas T cells are generated within the thymus, but are both thought to originate from a common lymphoid precursor. The individual receptors that they express have a unique antigen specificity determined by the sequence of the antigen-binding site. This is made up of variable (V) regions that are themselves composed of a V gene segment and a joining (J) segment, and in certain cases the diversity (D) segment also. These separate gene segments can be randomly assembled in order to generate V regions, that when fused to a number of

possible constant (C) regions form antigen receptors with almost unlimited specificities. Furthermore receptor diversity is also increased by other mechanisms including the random addition of nucleotides in the join between J and D segments by the TdT enzyme.

1.2.2 BCR diversity

The B cell antibody is formed from two separate polypeptide chains, heavy and light. There are two possible classes of light chain, λ and κ. In humans, the λ gene locus contains 30 V segments and 4 J segments as well as 4 possible C segments. In contrast, the κ locus contains 40 V, 5 J but only 1 C segments. The heavy chain is not only made up of 40 V and 6 J segments, but also includes 27 D segments. Once the full V region is assembled it can fuse with any of the possible C regions, generating more diversity. This dictates the nature of the Ig formed and also its subsequent function. For example fusion of the Cε region to the V region generates IgE but if the V region associates with Cμ, IgM is formed. The recombination of these gene segments is driven by the family of RAG enzymes. Further BCR diversity is promoted by somatic hypermutation, which involves the introduction of random point mutations into the V regions of the assembled heavy and light chains. During a B cell immune response, clones are preferentially selected that have improved antigen recognition through somatic hypermutation. This is called affinity maturation and occurs in the germinal centre regions. The possible combination of gene segments and other mutational mechanism that generate sequence diversity is thought to generate a possible $\sim 10^{14}$ Ig receptor antigen specificities.

1.2.3 B cell development

Initial B cell development occurs in the bone marrow, and can be subdivided into a number of different stages depending on the transitional phenotype of the cells. During the pro-B cell stages, the rearrangement of the D, H and J gene segments of the heavy chain locus occurs. Proliferation and survival of the pro-B cell is dependent upon cytokines such as IL-7 and also stem cell factor, both of which are derived from the bone marrow stromal matrix. Successful rearrangement of the heavy chain VDJ locus lead to the expression of the μ heavy chain that, in combination with a surrogate light chain, forms the pre-BCR. Cells expressing the pre-BCR are termed pre-B cells, and can signal for the heavy chain rearrangement to stop. After several rounds of pre-B cell proliferation, light chain assembly begins. The successful assembly of a light chain VJ segment leads to the expression of a completed IgM molecule on the B cell surface, generating an immature B cell. At this stage, any B cell expressing a self-reactive BCR is thought to be removed by the process of negative selection, thus ensuring that autoreactive B cells are eliminated. However, it has been suggested that B cells expressing an autoreactive BCR have further chances to rearrange the light chain and no longer be self-reactive, escaping deletion (Gay *et al.*, 1993). Immature B cells are then exported to the periphery, where they begin to express both IgM and IgD on the cell surface and recirculate till encountering antigen.

1.2.4 TCR diversity

The TCR, like the BCR, is composed of two separate protein chains, α and β, that are also the product of RAG mediated gene rearrangement events. In humans the α chain locus is composed of ~70 V segments and 61 J segments, whereas the β locus has 52 V, 13 J and 2 D segments. As for B cells, the addition of random nucleotides occurs in the join between V and D segment, contributing to diversity. However, in T cells, random nucleotide addition also occurs between the V and J segments of the TCRα gene. Furthermore, the TCRα chain locus contains many more J gene segments than the BCR locus, as this region has been shown to code for the CDR3 loop of the TCR that forms the antigen-binding region. While the BCR can undergo mutational changes outside the CDR3 region, this would be unfavourable to a TCR as it is required to bind to the conserved features of MHC molecules, thus greatly restricting the sites of mutational diversity.

1.2.5 T cell development

T lymphocytes are generated from pluripotent haematopoietic stem cells (HSC) that are found in adult bone marrow and foetal liver. It is thought that the HSC become common lymphoid precursors (CLP) that can become either T, B or NK cells (Ardavin *et al.*, 1993). Once inside the microenvironment of the thymus, interactions with the stromal cells and differentiating progenitor cells drive T cell development. For example Notch-1 ligation has been shown to play possible role in directing T cell development versus B cell formation as Notch-$1^{-/-}$ mice have B cell development in the thymus (Wilson *et al.*, 2001). Furthermore, it has been shown that the stromal

cells in the thymus express Notch ligands such as Delta-1 (Pui *et al.*, 1999; Radtke *et al.*, 1999). The stromal cell-derived cytokine IL-7 also plays an important role in driving T cell development as IL-$7^{-/-}$ mice show a block at the DN stage of T cell development (Maeurer and Lotze, 1998). In terms of *in vitro* B cell development it is possible to simply mimic this using a bone-marrow-derived stromal cell line and defined cytokines, however *in vitro* generation of T cells requires that the intricate microenvironment of the thymus is preserved. This has led to the use of whole foetal thymic organ cultures (FTOC) systems. Using this model it has been possible to analyse several aspects of thymocyte development, including the differentiation potential of various progenitors within the thymus, the importance of different stromal cell types in supporting this, and also it has been possible to manipulate the nature of signals being delivered (Zuniga-Pflucker, 2004).

The first stage of T cell development is the DN population, which can be subdivided into 4 populations based on the CD25 and CD44 expression profiles. During CD25 upregulation at the DN1 stage, cells will begin to rearrange and express the TCR genes. Cells that successfully rearrange γ and δ genes express the γδ TCR and become cells of the γδ lineage. Interestingly, IL-7 may also play a role here as overexpression of IL-7 seems to preferentially drive cells to the γδ lineage (Maeurer and Lotze, 1998). However those cells that successfully rearrange the β chain locus are able to form a functional pre-TCR with the pre-Tα chain and undergo β selection, making the transition from $CD25^{-}$ $CD44^{+}$ DN3 cells to $CD25^{-}$ $CD44^{-}$ DN4 cells. At the DN2 to DN3 stage, the TCRβ chain locus is thought to undergo allelic exclusion

and is no longer able to rearrange, ensuring that only one TCR specificity is generated per-cell, and the rearrangement of the α chain begins at the DP stage.

During the next stage of T cell development after successful TCR assembly thymocytes express both CD4 and CD8 co-receptors to form DP cells. These thymocytes are then subjected to positive and negative selection by testing the recognition capabilities of the TCR repertoire on self-pMHC complexes. The process of positive selection involves the selection of those cell bearing TCR able to recognise and bind self-MHC. In contrast cells expressing a TCR that either interacts with self-pMHC with extremely low affinity or not at all fail to signal these cells to survive and they die by neglect (Huesmann *et al.*, 1991). However, cells that recognise and bind self-pMHC with very high affinities may represent autoreactive T cells, therefore negative selection also exists to trigger these cells to undergo apoptosis. The net result of positive and negative selection is that only cells expressing a TCR that recognise a self-MHC with a relatively weak affinity are selected. It was considered that the self-peptide repertoire expressed within the thymus would be limited, and that antigens expressed by extra-thymic tissue sites in the body may not be present. This would have a dangerous outcome, as this model would predict that potentially autoreactive T cells clones specific for these tissue antigens would escape deletion and exit the thymus. However, a recently identified transcription factor termed AIRE has been shown to regulate the ectopic expression of peripheral tissue antigens in thymic stromal cells (Anderson *et al.*, 2002). Furthermore it has been possible to visualise the movement of progenitor cells

through the microenvironment of the thymus. This work has shown that HSC enter via the cortico-medullary junction, where they then undergo DN transition in the outer cortex and enter the medulla for the final stages of maturation (Lind *et al.*, 2001). Moreover, it seems that the specialised cells in these thymic areas may be essential to the particular developmental stages that occur there (Porritt *et al.*, 2003; Prockop *et al.*, 2002).

1.2.6 Lineage commitment

The final decision that a developing thymocyte has to make is whether to become a CD4 or CD8 cell. Early models of lineage commitment suggested that it was largely a stochastic event, in that the decision to downregulate CD4 or CD8 was random, and survival of the subsequent T cell relied on the fact the TCR was matched to the particular co-receptor (Jameson *et al.*, 1995). However recent studies have suggested that the nature of signal delivered to the developing T cell may actually regulate the lineage decision, and not simply the class of MHC it is able to recognise. For example, expression of chimeric molecules composed of the extracellular portion of CD8 fused to the cytoplasmic region of CD4 diverted a MHC-class I restricted TCR to the CD4 lineage (Itano *et al.*, 1996).

1.2.7 Peripheral lymphocyte functions

After development within their respective niches, mature lymphocytes patrol the periphery to detect the presence of infectious agents. Lymphocyte clones with unique antigen specificities are normally present at low frequency, however upon

encountering the specific antigen they undergo clonal expansion in order to expand the number of antigen-specific cells. During this period of proliferation, lymphocytes acquire a number of cell-type specific effector functions that serve to attack and neutralise the infecting agent.

1.2.8 B cell functions

When a B cell encounters specific antigen, it differentiates into a plasma cell and begins to secrete antibodies that are able to carry out several important immune functions. B cell activation can occur in one of two ways. Firstly, if a B cell binds its antigen, it can internalise and process the protein in order to express antigenic fragments on surface bound MHC class II molecules. The B cell is then able to present this peptide antigen to activated Th2 CD4 cells, which can then contribute to the activation status of the B cell. This kind of B cell activation is known as a T cell dependent stimulation. The Th2 CD4 cells expresses CD40L that can bind to CD40 on the B cell surface and, in conjunction with the CD4-derived cytokines IL-4 and IL-13, induce B cells to switch the Ig isotype they produce. This is an important mechanism as each type of Ig class has very different immunological function. For example, IgA is a potent neutralising antibody as it is soluble in body fluids and can bind to pathogens to block entry into cells. In contrast IgG1 is a powerful opsonising antibody as it can bind to the surface of the pathogen and aid phagocytic recognition through Fc receptors expressed by macrophages. Antibodies such as IgM are potent activators of the complement system and also mediate pathogen destruction. These B cell-T cell interactions occur in specialised structures called germinal centres (GC)

and facilitate the process of somatic hypermutation. B cells can also become activate independently of T cells by pathogen-derived polysaccharides, and these are called T cell independent antigens.

1.2.9 Naïve, effector, and memory CD4 T cells

T cells that express the CD4 co-receptor normally recognise extracellularly derived peptides in the context of MHC class II molecules only expressed by professional APC. It is thought that naïve T cells are located primarily in secondary lymphoid sites, including the LN, and Spleen. The number of naïve T cells is maintained by a balance between cell loss, homeostatic proliferation and new thymic emigrants (Rocha *et al.*, 1989). Upon encountering antigen, CD4 cells begin to differentiate into two different effector subsets (Seder and Ahmed, 2003). Th1 cells are important for protection against intracellular pathogens, and are characterised by their ability to produce cytokines such as IFNγ. At the transcriptional level, Th1 differentiation is thought to be controlled by STAT4 and T-bet. It is thought that Th1 cells are generated, in part, by the influence of the APC. For example an APC activated by a bacterial product such as LPS binding to the CD14 / TLR4 complex will induce high levels of IL-12. IL-12 has been shown to favour Th1 differentiation as it can induce IFNγ production in T cells. Th1 cells can also express surface molecules such as CD40L that can activate macrophages to become antimicrobial by producing NO and help to clear intracellular pathogens. In contrast to Th1 cells, Th2 CD4 cells produce cytokines such as IL-4, IL-5, and IL-13. IL-4 has been shown to silence the IFNγ locus, thus promoting Th2 development. Furthermore, the transcription factors

STAT6 and GATA-3 have been shown to be important to Th2 development. Th2 CD4 effector cells are often found in B cell rich areas of the body, such as the spleen, and are able to induce B cells to undergo Ig class switching to IgE by virtue of IL-4 and IL-13, as well as CD40L binding to CD40 on the surface of the B cell (Vercelli, 1995). The differentiation of CD4 cells to the Th1 or Th2 lineage is probably influenced by the initial cytokine milieu as a result of a specific antigen interacting with components of the innate immune system including DCs, macrophages and also possibly NK cells. After successful clearance of the pathogen, it is considered that a proportion of antigen experienced CD4 cells survive and become resting memory cells, which are able to mount a more rapid response upon secondary challenge It is thought that the long-term survival of memory CD4 cells relies on signals delivered to the T cell via IL-7, which also maintains memory cell numbers by driving homeostatic proliferation (Seddon *et al.*, 2003).

1.2.10 Naïve, effector and memory CD8 T cells

T cells that express the CD8 co-receptor normally recognise intracellularly derived peptide fragments that are presented by ubiquitously MHC class I molecules. On encountering antigen CD8 cells undergo a more rapid proliferation programme that CD4 cells (Seder and Ahmed, 2003), and begin to express a number of specialised genes that turn them into potent antiviral cells. The general function of CD8 effector CTL is to recognise and bind to any cell that is expressing cognate antigen and facilitates their destruction. To do this the CTL uses a protein called Perforin to punch holes in the target cell membrane and then deposits potent pro-apoptotic

molecules into the interior. CTL also express death receptor ligands such as FasL that bind to Fas on the surface of target cells and also induce apoptosis. As for CD4 cells, antigen experienced CD8 cells also form resting memory cells that are able to respond vigorously to antigen re-encounter. It is thought that IL-7 and IL-15 can contribute to CD8 memory generation and survival (Schluns and Lefrancois, 2003). There has also been some suggestions that the presence of CD4 cells greatly influences the quality of CD8 memory cells generated by immune challenge (Janssen *et al.*, 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). It is thought that memory CD8 cells are derived from effector cells as it has been suggested that the resulting memory pool size can be related back to the size of the effector pool (Hou *et al.*, 1994) and also gene chip profiling has also suggested a linear developmental pathway from naïve to effector then to memory CD8 cells (Kaech *et al.*, 2002).

1.2.11 Other thymically derived lymphocytes

As well as conventional αβ T cells, the thymus also supports the development of NK T cells and γδ T cells. NK T cells are also αβ TCR^+ but express a limited receptor diversity and also express the NK1.1 marker. Their development and activation is supported by an MHC class I variant molecule called CD1d, which is expressed on DC and monocytes and present glycolipid antigens to the NK T cells. NK T cells have been implicated in influencing the polarisation of CD4 cells to Th1 or Th2 differentiation, as they can produce polarising cytokines after activation (Godfrey and Kronenberg, 2004). T cells from the γδ lineage arise from DN cells that successfully rearrange the γδ T cell locus. It is thought that the first wave of γδ T cells become

dendritic epidermal T cells and are found in the epidermis, and the second wave of γδ T cells populate the reproductive tract. Both of these early waves have limited receptor diversities, however in later waves this changes. The role of γδ T cells is not clear, however some subsets may be responsible for removing damaged gut epithelial cells.

1.2.12 MHC structure and function

MHC molecules are effectively the focal point of the adaptive immune system as they can provide both survival and stimulatory signals to T cells. MHC molecules are also able to bind and display an amazing repertoire of peptide fragments derived from many sources.

1.2.13 MHC gene loci

The genes encoding the MHC class I and II molecules are highly polygenic as they are encoded by several different sets of genes arranged into clusters in the genome. Secondly, these MHC genes are highly polymorphic and two molecules from the same MHC genotype can differ by up to 20 amino acids. Therefore any given individual can produce and express a diverse repertoire of MHC molecules, as they are also normally heterozygous for any given MHC haplotype. The polymorphisms have been shown to occur at specific sites responsible for forming the peptide-binding cleft and also the T cell recognition sites, and are able to increase the possible number of peptides any given MHC genotype can bind. This is an important contribution to an immune response, as specific T cell clones are only able to

recognise peptides expressed by specific MHC genotypes; this is called MHC restriction. Without the polymorphisms, immune recognition may be less effective as the T cell clone may not be able to recognise the MHC genotype that was able to bind its cognate antigen.

1.2.14 MHC structure, loading and presentation

MHC class I molecules are almost ubiquitously expressed, reflecting the fact that almost any cell may require elimination by CTL. The MHC class I molecule is formed from two polypeptide chains. The α chain is derived from the MHC gene locus and is folded into 3 domains and is associated with the non-variant β micro-globulin protein. The α1 and α2 regions form the peptide binding cleft and can bind peptide of 8-10 amino acids in length. In general, these peptides are derived from the cytosol and are often viral-based products that are degraded in proteosomes. Interestingly, two components of the proteosome, LMP2 and LMP7 are encoded within the MHC gene cluster, and like MHC class I molecules, can be induced by IFN. Newly produced MHC class I polypeptides are found within the ER in an unfolded state and a number of chaperon proteins then assist in the assembly of the α chain, β_2 micro-globulin and peptide. These chaperons include calnexin, which helps to retain the partially assembled MHC molecules in the ER allowing the β_2 micro-globulin to bind and calreticulin, which is a chaperon that keeps the MHC class I stable to then receive the peptide. The TAP1 and TAP2 proteins are then responsible for loading the MHC with peptide. Only when the MHC class I molecule is assembled with peptide can it be expressed on the cell surface.

The MHC class II molecule is formed from two polypeptide chains called α and β, both of which are encoded by the polymorphic MHC loci. The peptide-binding cleft is formed from the α1 and β1 domains. Unlike class I molecules, MHC class II molecules are not restricted in the size of peptide they can bind, possibly because the peptide binding domain is comparatively in a more open conformation. These peptides are often derived from endosomes that degrade certain intracellular proteins and also protein that enter cells by endocytosis. While in the ER and undergoing assembly, MHC class II molecules are prevented from binding peptide prematurely by the invariant chain protein that blocks access to the peptide binding groove. When assembly is completed the MHC class II molecules are transported to the endosomal compartment and the invariant chain is cleaved by protease to form the CLIP that remains in the peptide binding grove. The MHC class II-like protein HLA-DM is found in the endocytic compartment and is able to stabilise the MHC class II molecules and mediate the release of CLIP, allowing peptides to bind.

MHC class II molecules are expressed by professional APC such as DCs, macrophages and B cells. APC are responsible for immune surveillance as they expressed in nearly all tissues in various guises. For example Langerhan cells are found in the epithelium and upon encountering antigen, migrate to the draining lymph nodes and mature into potent APC that are able to activate T cells. Several studies have suggested that the polarisation of an immune response (e.g. Th1 or Th2 CD4 polarisation), is largely dependent upon the status or type of APC. For example an APC that has been activated through TLR4 and LPS will produce high levels of IL-12

that will promote CD4 T cells to become Th1. Furthermore, it has been suggested that DCs activated by CD40-CD40L interactions are able to then activate CD8 T cells to become more effective CTL (Bennett *et al.*, 1998).

1.2.15 Cross presentation

Although it was originally considered that the MHC class and antigen source was a fixed rule, studies have also suggested that MHC class I molecules can present peptides that are derived from extracellular sources. It is thought that phagosomes can fuse with ER vesicles to form hybrid compartments that contain the components of class I assembly and newly synthesised class I MHC molecules. Then phagocytosed extracellular proteins are then degraded by the phagosomes and can be presented on class I molecules. Cross-priming may be functionally important to activate a CTL response when the virus would not normally infect the DC itself (Heath *et al.*, 2004)

1.3 Mechanisms of immune regulation: success and failure

While the thymus is clearly the major organ for regulating tolerance against self-antigens through the function of the AIRE transcription factor, this does not overcome the problems associated with the many commensal bacteria that reside in the GI tract. These antigens are not present in the thymus during negative selection and therefore potentially reactive T cells will escape deletion. Therefore, there are a

number of peripheral mechanisms to control the activation of potentially autoreactive T cells.

1.3.1 Regulatory T cell subsets

Classically, T_{reg} cells were identified by their ability to suppress the activation of naïve T cells both *in vitro* and *in vivo*. So-called Th3 cells have been shown to secrete high level of TGFβ and were originally thought to be induced by low-dose antigen feeding to establish oral tolerance, and also favoured by a Th2 cytokine milieu (Strober *et al.*, 2002). In contrast, the Tr1 cell is thought to function by secreting large amounts of the anti-inflammatory cytokine IL-10 and can be induced by IL-10 itself and also IFNα. Lastly, the CD4 $CD25^+$ T_{reg} cell is thought be thymically derived and inhibits effector T cell by cell-cell contact mechanisms that are possibly mediated by surface bound TGFβ (Nakamura *et al.*, 2001). In situations of strong antigenic stimulation, these cells may also be able to secrete IL-10 and thus share properties of both Th3 and Tr1 regulatory cells. It is also possible that NK T cells have some suppressive function as the CD1d molecule that they recognise is found abundantly on epithelial cells and NK T cells have been shown to produce IL-10 (Godfrey and Kronenberg, 2004).

1.3.2 Anergy

In situation where a T cell encounters antigen without the delivery of the appropriate co-stimulatory signal, a state of subsequent non-responsiveness called anergy ensues

(Schwartz, 2003). This may be an important mechanism of ensuring tolerance to tissue antigens as the cells presenting these peptide to T cells often do not express the necessary co-stimulatory molecules. Anergised T cells are thought to have undergone a number of phenotypic changes including the down-modulation of co-stimulatory receptors, and are subsequently unable to produce IL-2 even in response to full activation signals (Schwartz, 2003). It is unclear why these anergic autoreactive cells are often maintained *in vivo*, however they may serve to out- compete other potentially autoreactive naïve cells that have not yet been activated. Interestingly, CD8 cells have been reported to undergo a form of anergy called AINR. This has been shown to differ from classical anergy as it can be induced after full T cell activation (Deeths *et al.*, 1999). The purpose of such a mechanism may be to limit the duration of a CTL response (Tham *et al.*, 2002). It is also thought that T cells will become anergic if continually exposed to antigen *in vivo* during the course of chronic infections. This has been shown in a mouse engineered to express the peptide epitope of LCMV, in that transferred LCMV-specific CD8 cells rapidly became anergic (Ehl *et al.*, 1998).

1.3.3 AICD

Once a T cell becomes activated and the infection is neutralised, it is important to then curtail the immune response as prolonged effector function can prove harmful to self. One way in which this is achieved is through the elimination of activated T cells. It is thought that AICD involves the selective removal of pre-activated T cells by apoptosis-inducing mechanisms. This is thought to occur via the ligation of death

receptors on the surface of activated T cells that include Fas and also TNFα (Lenardo *et al.*, 1999). Furthermore, AICD may also link with anergy, as studies have shown that activation through the TCR alone may also induce T cell apoptosis (Shi *et al.*, 1989). As well as active mechanisms to remove effector T cells, there are also death receptor independent methods (Lohman *et al.*, 1996) that may act through the downregulation of the anti-apoptotic molecule Bcl-2 (Strasser *et al.*, 1995). It is thought that Bcl-2 expression may be regulated by pro-survival cytokines, therefore the withdrawal of these cytokines may also result in T cell death. This mechanism is thought to regulate AINR mediated control of CTL responses as the CD8 cells die through a lack of IL-2 (Tham *et al.*, 2002).

1.3.4 Autoimmunity

In situation where there is failures in the mechanism of tolerance, the attention of the immune system can turn toward self. This can lead to extensive tissue destruction at sites within the body by disregulated Th1 mediated inflammation, CTL function, and by inappropriate B cell activation by T cells. For example, type I diabetes is caused by autoreactive cells specific for the pancreatic β cell antigen destroying these cells, leading to a loss of insulin production. Furthermore, arthritis is thought to arise from uncontrolled inflammation of the synovial membranes of the joint, driven by a disregulated Th1 immune response. Interestingly, a number of immune disorders have been shown to also have strong association with specific HLA-subtypes. For example patients suffering from ankylosing spondylitis have been shown to express the potentially novel MHC-class I subtype, HLA-B27 (Reveille *et al.*, 2001), which

can be expressed as a homodimer without the β_2 microglobulin. It is thought that the structure of HLA-B27 may contribute toward how the immune system recognises this MHC class I subtype (Allen and Trowsdale, 2004; Allen *et al.*, 2001). Also HLA-DR4 has been shown to increase the susceptibility to rheumatoid arthritis.

Defects in AICD may also contribute to autoimmune disorders. The evidence for this comes from the *gld/gld* mice that lack functional FasL and the *lpr/lpr* mice that do not express active Fas. Both mice suffer from systemic autoimmune disorders due to an inability to eliminate activated T cells (Suda *et al.*, 1993; Watanabe-Fukunaga *et al.*, 1992). Fas mutations have also been found in humans suffering from general lymphoproliferative autoimmune disorders (Fisher *et al.*, 1995).

It is not only failures in peripheral tolerance mechanisms that can lead to autoimmunity. The AIRE protein was first identified as the mutated gene in humans suffering from a multi-organ autoimmune disorder (Anderson *et al.*, 2002). Furthermore, $AIRE^{-/-}$ mice also suffer from autoimmune pathology, highlighting the importance of the thymus in regulating peripheral tolerance (Anderson *et al.*, 2002).

1.4 Peripheral T cell signalling: from TCR to nucleus

One common question that arises is exactly how T cells are able to translate signals delivered through the TCR into several different functional outcomes. What is clear is that activation of Lck and Fyn is one of the earliest biochemical events detected

upon TCR ligation. These are then responsible for transducing signals from the TCR to the nucleus through a number of signalling networks (see Figure 1.1)

1.4.1 Models for TCR engagement and activation

There are two models that have been proposed to explain how T cell activation is initiated via the TCR. The first is the serial engagement model and is based on the idea that when a T cell encounters an APC it requires that a certain number of TCR molecules are engaged by pMHC molecules in order to initiate signalling (Lanzavecchia and Sallusto, 2000). The problem with this model is that an APC will present both foreign and self-pMHC to a TCR. The second model is an advancement of the first in that the T cell does not simply activate after a certain number of pMHC engagements, but it is able to scan the APC and distinguish between self-pMHC and foreign pMHC molecules. This is termed the kinetic proofreading model (McKeithan, 1995). In the second model, although self-pMHC is expressed at ~1000-fold higher density than foreign peptide fragments, it is thought that TCR will bind self-pMHC with ~10-fold lower affinity than foreign pMHC. Therefore only high affinity pMHC interaction will stimulate the T cell. However a TCR must strike a balance between binding avidity for foreign pMHC and the ability to disengage the limiting number of foreign pMHC, allowing them to trigger further TCR molecules. There are data that conflicts with the proofreading model in that a mutant high-affinity TCR showed no defect in triggering to limiting foreign pMHC (Holler *et al.*, 2001).

1.4.2 IS formation and cytoskeletal arrangements

In a resting state, microscopic analysis of the T cell reveals that it is a small sphere with antigen and adhesion receptors distributed evenly on its surface. However T cells must be motile and be able to move toward agents such as chemoattractants that are produced by innate immune cells and signal potential sites of infection. They do this by forming actin polymer structures that push the T cell membrane forward, allowing the cell to move. Then upon encountering antigen the T cell alters its shape to reorientate itself to form a contact with the presenting APC. This involves the formation of flat sheet-like ruffles termed lamelipodia, spikes called filopodia and larger projections termed pseudopodia. In terms of signalling molecules, 30-40% of the TCRζ chain is physically linked to the cytoskeleton, as well as other molecules including MHC class I, CD2, CD4, CD8, LFA-1, CD28 and CD44. Therefore, upon activation these receptors are recruited to the actin cytoskeleton and form a cluster at the T cell-APC immunological synapse (IS) called a supra-molecular activation complex (SMAC). This is an important event in activation as it brings signalling molecules into proximity with one another and increases the potential density of signal. This can be visualised by staining with the mAb 4G10, revealing that the APC - T cell contact site is rich in pTyr moieties. Certainly, treatment of cells with agents that block cytoskeletal rearrangements such as cytochalasin D also prevent T cell activation. It has been described that the arrangement of these molecules is highly ordered in that the TCR, CD2, and CD28 form part of a central cluster called the cSMAC, whereas molecules such as LFA-1 are excluded into a peripheral ring, or pSMAC. Comparatively larger structures such as CD45 are completely excluded

from the SMAC. These structures have been noted in Th cells, Tc cells and NK cells. Lck may be actively brought to the cSMAC by virtue of its association with CD4 and CD8, both of which are tethered to the cytoskeleton. Moreover, Lck may be important to the association of phosphorylated CD3ζ with the cytoskeleton (Rozdzial *et al.*, 1998).

There are a number of molecules that have been shown to regulate cytoskeletal events, and deficiency in these molecules often abrogates T cell activation. For example VAV deficient T cells are defective in synapse formation and cytoskeletal rearrangement in response to APC stimulation. Interestingly Fyn has been implicated as the kinase responsible for VAV phosphorylation (Huang *et al.*, 2000). Another important set of proteins that plays a role in cytoskeletal modelling is the ADAP/SLP-76 complex. This complex has been shown to contain Nck, VAV and WASp. The WASp protein activates the Arp2 complex that can act as the nucleating centre for actin, allowing the formation of F-acting polymers to occur. Interestingly humans deficient in the WASp protein have T cells that have defects in cytoskeletal organisation. Recent work has suggested that WASp phosphorylation is defective in the absence of Fyn. Furthermore, Fyn has also been shown to selectively phosphorylate the FAK family member Pyk2 (Qian *et al.*, 1997), which is another family of proteins involved in cytoskeletal regulation. Moreover, ADAP binding to SLP-76 has been shown to be essential to the clustering of LFA-1 molecules on T cells (Wang *et al.*, 2004), and Fyn has been implicated as the kinase responsible for phosphorylating ADAP, allowing SLP-76 to bind (Raab *et al.*, 1999). However it

should also be noted that the initiation of T cell signalling may occur before the formation of a mature IS, as it has been reported that although the TCR moves from the pSMAC to form part of the mature IS, Lck is only ever detectable in the pSMAC (Lee *et al.*, 2002).

1.4.3 GEMs and TCR signalling

It has been hypothesised that the localisation and partioning of signalling molecules are essential to their function. Proteins that are membrane localised are thought to be regulated in-part by the presence of specialised lipid domains called glycolipid-enriched microdomains (GEMs). These domains were first identified by their insoluble nature in cold, non-ionic detergents, as normal cell membrane components will dissolve in these conditions. It is possible to isolate GEMs using sucrose gradients due to their comparative buoyancy. It is thought that GEMs act as environments whereby signalling molecules can be brought together to perpetuate signals. Proteins can be recruited to GEMs by modifications such as GPI linkage and N/S-acetylation. Key signalling molecules such as LAT are present within these domains, acting as a scaffold for further molecules that are recruited to the GEM following activation. It is possible to isolate GEM from unstimulated and stimulated cells and detect gross differences in the proteins present. Interestingly, Lck and Fyn are both present in GEM by virtue of acetylation. However, in a resting cell the majority of Lck is located outside GEM due to co-receptor association, furthermore those molecules present in the GEM are in an inactive conformation possibly due to the presence of LAT (Kabouridis, 2003). In contrast, Fyn is thought to be ever

present within the GEM (Yasuda *et al.*, 2002) and upon activation, it is thought that co-receptor associated Lck translocates to lipid rafts, possibly to increase Fyn activity (Filipp *et al.*, 2004; Filipp *et al.*, 2003).

1.4.4 Co-stimulatory T cell receptors

Ligation of the TCR alone is often considered to be insufficient for full T cell activation and can lead to an anergic state (Schwartz, 2003). Expression of B7.1 and B7.2 on the presenting APC has been shown to activate the CD28 co-receptor expressed by T cells (Azuma *et al.*, 1992) and deliver the so-called signal 2 to the T cell. CD28 has been shown to signal through PI3-K, ITK and Grb2, and interestingly, Fyn and Lck have been suggested as the kinases responsible for recruiting these molecules to the cytoplasmic tail of CD28 (Raab *et al.*, 1995). CD28 derived activation of PKB has been implicated in the regulation of cytokine production by NF-κB (Kane *et al.*, 2002). CD28 signalling may also activate the Jnk family of MAPK, leading to the modulation of c-Jun expression and function. More classically, CD28 signalling has also been shown to upregulate IL-2 production by increasing the stability of IL-2 mRNA, but may also upregulate *Il2* transcription directly through the CD28RE within the *Il2* gene (Shapiro *et al.*, 1997)

The SLAM receptor is a member of the CD2 superfamily and is upregulated on activated T cells (Cocks *et al.*, 1995). It is thought that SLAM may modulate the immune response by regulating cytokine production and also possible CTL function (Henning *et al.*, 2001). For signalling purposes the cytoplasmic tail associates with

the SAP adapter molecule that has been shown to be mutated in human X-linked immunoproliferative disease (Davidson *et al.*, 2004). SAP has been shown to bind to and recruit Fyn to the SLAM receptor, and Fyn is essential for phosphorylation of SLAM (Chan *et al.*, 2003). Interestingly, SLAM signalling has been shown to couple with PKCθ mediated NF-κB regulation (Cannons *et al.*, 2004; Davidson *et al.*, 2004).

1.4.5 Signal regulation and termination mechanisms

Once a signal has been initiated, there is also a need for it to be terminated as and when the invading agent has been neutralised. Furthermore, naïve peripheral T cells also require TCR engagement by self-pMHC molecules in order to survive (Seddon and Zamoyska, 2002). Without appropriate regulation of T cell signalling at the molecular level, a given response against a foreign or self-antigen may become perpetuated and harmful to the host, leading to autoimmune disorders.

1.4.6 Phosphatases

In general the addition of phosphate groups to amino acid residues in proteins is thought to promote signal transduction, therefore the removal of these moieties by phosphatase enzymes is thought to counteract this. PTEN is a phosphatase able to catalyse the conversion of the PKB activating lipid moieties PIP_3 into PIP_2 and then into PIP (Seminario and Wange, 2003), thus antagonising PI3-K function. This leads to a change in the membrane lipid constituents affecting PH domain containing proteins, and also may affect PLCγ1 activation due to breakdown of the PIP_2 substrate. Another phosphatase that metabolises PI3-K lipid products is SHIP,

converting PIP_3 to a PIP_2 moiety that is distinct from the type generated by PTEN. Interestingly, $SHIP^{-/-}$ mice develop severe multi-organ autoimmunity, underscoring the importance of this molecule in immune regulation. A further important T cell phosphatase is SHP-1. It has been shown to also antagonise PI3-K signalling by dephosphorylating Tyr688 within the SH2 domain of the p85 subunit (Seminario and Wange, 2003). Interestingly, it has also been suggested that SHP-1 may be able to dephosphorylate the ITAM residues in the CD3 complex (Raab and Rudd, 1996). The fact that so many phosphatases target PI3-k underscores the importance of this signalling molecule in the regulation of several pathways.

1.4.7 ITIM-containing receptors

Receptors that contain ITIMS have also been shown to regulate T cell activation. ITIMs are similar to an ITAM but tend to recruit phosphatases that serve to downmodulate signalling. An example of an ITIM containing receptor is PECAM-1. It has been shown to recruit SHP-2 and attenuate TCR signalling. Interestingly Lck has been implicated in the phosphorylation of the PECAM-1 ITIM, as it was undetectable in Lck deficient Jurkat cells (Newman *et al.*, 2001). Another ITIM containing receptor is PD-1, and $PD\text{-}1^{-/-}$ mice suffer from an autoimmune disorder (Zha *et al.*, 2004) underscoring the role of these receptors in controlling peripheral tolerance.

1.4.8 CTLA-4

Co-stimulatory CD28 signals are antagonised by the B7 family member CTLA-4 (Linsley and Ledbetter, 1993), which may also contain an ITIM motif. CTLA-4 binds to CD28 with higher affinity than B7.1/2 and can associate with the phosphatase SHP-2. Mice lacking functional CTLA-4 suffer from an autoimmune syndrome (Tivol *et al.*, 1995). Interestingly, a number of studies have implicated Lck and Fyn as the kinases responsible for phosphorylating the tyrosine residues important for SHP-2 binding and trafficking of CTLA-4 to the cell surface (Hu *et al.*, 2001; Chuang *et al.*, 1999; Miyatake *et al.*, 1998; Marengere *et al.*, 1996).

1.5 TCR signalling molecules and pathways

The TCR is composed of α and β polypeptide chains that confer antigen specificity. These are coupled to the three invariant chains of the CD3 complex, arranged as a heterodimers of $\varepsilon\delta$ and $\gamma\varepsilon$. The CD3 complex is further associated with the $\zeta\zeta$ homodimer. The CD3/ζ complex contain motifs know as ITAMS ($Yxx[L/V]x_{6-9}Yxx[L/V]$, where Y is tyrosine, L is leucine, V is valine and x can be any residue. When these ITAMS are phosphorylated they form docking sites for the ZAP-70 tyrosine kinase, facilitating its activation also by phosphorylation. Studies have suggested that Lck may be essential to this step, as in the Lck deficient JCaM1 cell line, phospho-ζ induction, ZAP-70 recruitment and ZAP-70 phosphorylation were all defective in response to TCR signals (Williams *et al.*, 1997b; al-Ramadi *et al.*, 1996;

Gupta *et al.*, 1994; Straus and Weiss, 1992). The defective ZAP-70 activation could be restored by transfecting back Lck (Straus and Weiss, 1992). It has been suggested in Jurkat cell lines transfected with either Lck or Fyn, that Fyn is capable of mediating a distinctive pattern of ITAM phosphorylation (Denny *et al.*, 2000). It is thought that ZAP-70 can bind to doubly phosphorylated ITAM motifs, but also it has been suggested other signalling molecules such as Shc (Osman *et al.*, 1995), PI3-K (Exley *et al.*, 1994) and Src kinases themselves (Johnson *et al.*, 1995) can bind singly phosphorylated ITAMS. Once activated by phosphorylation of Tyr-493 in its activation loop by Lck (Chan *et al.*, 1995), but possibly not Fyn (Denny *et al.*, 2000), ZAP-70 is able to phosphorylate LAT (Wardenburg *et al.*, 1996) and SLP-76 (Zhang *et al.*, 1998b).

1.5.1 LAT

LAT is a transmembrane adapter protein that contains two cysteine residues that when palmitoylated, target this protein to the plasma membrane (Lin *et al.*, 1999; Zhang *et al.*, 1998a). LAT contains a long cytosolic region containing 9 Tyr residues that can be phosphorylated to form a scaffold for binding several proteins. These include Grb2/SOS, GADS and PLCγ1 (Zhang *et al.*, 2000). Interestingly, Lck has been shown to co-precipitate with LAT (Kabouridis, 2003). Moreover, these particular Lck molecules were shown to be in a closed inactive conformation and this was dependent on the co-localisation of LAT with Lck in lipid rafts. The second target of ZAP-70 phosphorylation, SLP-76 is also recruited to this complex by virtue of its association with GADS (Ishiai *et al.*, 2000). SLP-76 also binds ITK, which

may assist in the activation of LAT associated PLCγ1 (Su *et al.*, 1999), and also molecules such as ADAP (Wang *et al.*, 2004), VAV and Nck (Bubeck Wardenburg *et al.*, 1998) that link into pathways regulating rearrangement of the actin cytoskeleton and cell adhesion. Interestingly, Fyn has been implicated in the phosphorylation of both ADAP (Raab *et al.*, 1999) and VAV (Huang *et al.*, 2000).

1.5.2 PLCγ1

Activation of the PLCγ1 enzyme is another important step in T cell activation. Once activated, possibly by ITK-mediated phosphorylation, it catalyses the hydrolysis of the lipid moiety PIP_2 into IP_3 and DAG (Nishibe *et al.*, 1990). However there are also reports that Lck and Fyn can associate with PLCγ1 directly (Weber *et al.*, 1992), and have been shown to phosphorylate PLCγ1 *in vitro* (Liao *et al.*, 1993). Furthermore, VAV may also be important to the activation of PLCγ1 as $VAV^{-/-}$ DP thymocytes show defects in calcium mobilisation and PLCγ1 phosphorylation (Reynolds *et al.*, 2002).

1.5.3 IP_3, Ca^{2+} and NF-AT

Production of IP_3 leads to the release of Ca^{2+} from the ER by binding to IP_3 receptors located on the surface. Interestingly, it has been suggested that Fyn binds to theses IP_3 receptors and may phosphorylate them (Jayaraman *et al.*, 1996). The drop in intracellular Ca^{2+} includes the opening of the CRAC channels allowing entry of extracellular Ca^{2+} into the cell (Crabtree and Olson, 2002). The increase in intracellular calcium has been shown to lead to the activation of the Ca^{2+} /calmodulin-

dependent phosphatase calcineurin (CN) (Aramburu *et al.*, 2000). Once activated this has been shown to positively regulate the translocation of NF-AT family members to the nucleus to drive gene transcription. CN functions by dephosphorylating a number of serine residues in the N-termini of NF-AT members revealing the nuclear localisation signal sequence (Klemm *et al.*, 1997). There are a total of 5 NF-AT members and NF-AT1 ($NF\text{-}AT_p$) and NF-AT2 ($NF\text{-}AT_c$) are essential to the expression of immune regulator genes including IL-2, IL-4, CD40L and FasL (Peng *et al.*, 2001). NF-AT nuclear translocation is antagonised by GSK3 as it can rephosphorylate the serine residues masking the nuclear translocation signals (Beals *et al.*, 1997) and this has been shown to reduce IL-2 production (Ohteki *et al.*, 2000).

1.5.4 Ras and Erk

DAG, the other product of PIP_2 hydrolysis by PLCγ, has been shown to activate RasGRP (Ebinu *et al.*, 2000) and PKCθ (Isakov and Altman, 2002). RasGRP is a GEF, thought to be activated by DAG-mediated membrane translocation (Dower *et al.*, 2000). It can mediate the activation of the small G protein Ras by catalysing the conversion of GDP to GTP. When Ras is bound to GDP it is inactive, however when GTP is bound it is in an active conformation. Alternatively, Ras can also be activated by the PLCγ independent pathway involving another GEF called SOS. When SOS binds to the inducibly-phosphorylated adapter Grb2 and becomes membrane localised it becomes active and can also catalyse the conversion of GDP to GTP and activate Ras.

Once activated, RasGTP has been shown to activate the Erk MAPK cascade through the activation of the MAPKKK Raf. Raf in turn phosphorylates a serine residue in the MAPKK Mek1. Mek1 is then able to catalyse the dual phosphorylation of Tyr and threonine residues found in the activation segment of MAPK members. It is thought that in T cells the major MAPK activated by the Ras/Raf pathway is Erk1 and Erk2. Erk has been shown to be able to translocate to the nucleus where it can activate members of the Ets family of transcription factors such as Elk1 that has been shown to regulate the *c-Fos* gene, a component of the AP-1 transcription regulator. Also a recent paper has suggested that Erk and Lck can interact directly with one another (Stefanova *et al.*, 2003).

Recently, another TCR regulated pathway has been described that influences Erk activity. The molecule Shc has been shown to bind to singly phosphorylated ITAM residues within the ζ chain (Galandrini *et al.*, 1997). It is thought that Lck may be able to phosphorylate at least one Tyr residue in Shc (Walk *et al.*, 1998). Once phosphorylated, Shc can bind to Grb2/SOS and brings the whole complex to the membrane and activate the Ras/Raf/Erk cascade. It has been suggested that activation of Erk by Shc is important to the production of IL-2 by T cells by controlling the translocation of the NF-κB member c-Rel.

1.5.5 PKCθ and NF-κB

Another important target of the PIP_2 cleavage product DAG is PKCθ. In an inactive form PKCθ resides within the cytosol, however on binding DAG it translocates

rapidly to the cSMAC of the IS. PKCθ has been shown to regulate the function of NF-κB members by regulating IKK degradation through molecules such as CARMA1 and Bcl10 (Baier, 2003). When IKK is associated with NF-κB proteins it masks the nuclear translocation signal, therefore IKK degradation reveals this motif allowing NF-κB to enter the nucleus. Furthermore, PKCθ may also play a role in regulating NF-AT, possibly by regulating Gsk3 function via an association with PKB (Altman and Villalba, 2003). Finally PKCθ can also activate the Jnk pathway of MAPK (Ghaffari-Tabrizi *et al.*, 1999). Interestingly, it has been suggested that Lck can phosphorylate PKCθ in Jurkat cells (Liu *et al.*, 2000). Furthermore, it has recently been shown that Fyn deficiency may have a detrimental effect on PKCθ regulated NF-κB activation. $Fyn^{-/-}$ CD4 T cells show impaired PKCθ membrane translocation and are unable to degrade IKBα, resulting in defects in cytokine production (Cannons *et al.*, 2004).

1.5.6 Other MAPK pathways

Jnk activation is also an important event in T cell activation. Jnk is activated by MKK4/7 (Tournier *et al.*, 2001; Yang *et al.*, 1997), which in turn may be regulated by VAV and by PKCθ {Moller, 2001 #314}.. Once activated Jnk regulates the activity and expression of the c-Jun component of the AP-1 complex. Jnk exists as two different isoforms in T cells, and studies have suggested that Jnk1 and Jnk2 may have distinct functions in CD8 T cell activation (Conze *et al.*, 2002).

Another signalling circuit important to T cell function is the p38 MAPK pathway. It is thought to be activated by MKK3 and MKK6, which may be downstream of molecules such as Cdc42 and Rac. The p38 MAPK can regulate the function of transcription factors such as ATF2 that plays a role in the production of c-Jun. Interestingly a very recent paper has suggested Lck may play a role in phosphorylating Tyr 323 of p38 MAPK via ZAP-70 activation, and that this residue is important for TCR mediated p38 activity (Salvador *et al.*, 2005).

1.5.7 PI3-K and PKB

TCR activation can also induce the activity of PI3-K, possibly through the action of the adapter molecule Gab2 (Kane and Weiss, 2003). Interestingly, the SH3 domain of Lck has also been shown to bind a proline rich domain in the p85 subunit of PI3-K, possibly recruiting it to CD4 (Prasad *et al.*, 1993b), furthermore the SH3 domain of Fyn may also bind PI3-K at the same region (Prasad *et al.*, 1993a). PI3-K catalyses the formation of various phosphatidyl moieties including PIP_2, the PLCγ substrate, as well as PIP_3. This changes the lipid constituents of the membrane facilitating the recruitment of PH-domain containing proteins to the plasma membrane (Kane and Weiss, 2003). One such PH-domain protein is the serine/threonine kinase PKB. Activation of PKB is mediated by another PH-domain protein PDK1, that can phosphorylation Thr308 located in the kinase activation loop (Cantrell, 2002). Once activated, PKB can regulate a number of cellular events by virtue of its downstream targets. For example, it can positively regulate NF-AT nuclear translocation by phosphorylating GSK3 and inactivating this kinase (Cantrell, 2002). Moreover

expression of active PKB has been shown to increase production of NF-AT regulated cytokines including IL-2. PI3-K and PKB activation has also been shown to induce the E2F transcription factor that can regulate cell cycle progression and also c-myb, a regulator of cell survival (Cantrell, 2002). PKB may also regulate cell survival by phosphorylating and inactivating pro-apoptotic molecules such as BAD and caspase 9 (Seminario and Wange, 2003).

1.6 Src kinases

Src was first identified as a viral gene product of the Rous sarcoma virus that had tyrosine kinase activity and cell transformation potential (Bernstein *et al.*, 1976). Since then a total of 9 related Src family kinase, including Lck and Fyn, have been identified as signalling components in both non-transformed and transformed cells (Thomas and Brugge, 1997). Lck was first identified in the murine T cell lymphoma LSTRA, and was originally called the lskT gene (Marth *et al.*, 1985). Fyn (originally called Syn) was identified by two groups from human cDNA libraries by virtue of its sequence similarity with Src (Kawakami *et al.*, 1986; Semba *et al.*, 1986). Work from several labs has identified Lck and Fyn as key mediators of cell signalling, including T cells that express Lck as well as the exon 7B splice variant of Fyn called FynT (Cooke and Perlmutter, 1989). Furthermore, they can both shown transforming potentials when expressed in mutant forms in various cell types (Adler and Sefton, 1992; Semba *et al.*, 1990).

1.6.1 Src kinase structure

In terms of structure, each family member follows a strict molecular arrangement of protein domains (see Figure 1.2A). The SH1 (kinase), SH2 and SH3 (both protein-protein interacting motifs) are highly conserved between Src members. SH2 domains bind to pTyr residues in target proteins facilitating protein-protein interactions by virtue of the secondary sequence structure. These motifs are composed of a phosphotyrosyl recognition pocket that contains a conserved arginine residue that mediates electrostatic interactions with the target pTyr (Waksman *et al.*, 1992). However it is thought that substrate specificity of the SH2 domain is conferred by the divergent nature of the C-terminal recognition pocket (Kuriyan and Cowburn, 1997). SH3 domains of Src family kinases generally recognise and bind poly-proline motifs (RxxPxxP) in target molecules (Weng *et al.*, 1995). Interestingly, the SH3 domain of Fyn is an exception in that it can interact with the proline independent motifs (RKxxYxxY) found in SKAP55 (Kang *et al.*, 2000) and also to the SH2 domain of the adapter SAP (Latour *et al.*, 2003).

Furthermore, Src kinases possess SH4 domains that contain a run of between 50-70 residues known as the unique region that show no sequence similarities between family members. Within the SH4 region there are residues that can be mirystoylated, palmitoylated or s-acetylated to facilitate targeting to cellular membranes (Resh, 1999; Koegl *et al.*, 1994). The di-cysteine motif in the unique region of Lck has been shown to be essential for CD4/CD8 co-receptor binding (Shaw *et al.*, 1989; Rudd *et al.*, 1988; Veillette *et al.*, 1988). The crystal structure of inactive Src has been solved

(see Figure 1.2B) and shows the spatial arrangement of these domains (Williams *et al.*, 1997a). The solved structure showed that kinase domain is composed of two lobes, with the C terminal lobe containing the regulatory activation loop, which in concert with the cleft formed between the two lobes, mediates the phosphotransfer reaction (Taylor *et al.*, 1992).

1.6.2 Src kinase regulation

Src kinases, including Lck and Fyn can be regulated at several levels. For example, when phosphorylated on the Tyr residue located within the activation loop, the structure of the Lck catalytic domain has been shown to be held in an active conformation (Yamaguchi and Hendrickson, 1996). It is thought that phosphorylation of these residues in Lck and Fyn (see figure 1.2A) is mediated by transphosphorylation, however a recent study by Filipp and colleagues suggest that Lck may influence the phosphorylation of Tyr 417 in Fyn (Filipp *et al.*, 2004; Filipp *et al.*, 2003).

A second level of regulation is provided by the phosphorylation of the Tyr residue located in the C-termini of Src kinase molecules. This causes the SH2 and SH3 domains to turn inward and make intramolecular association that close the catalytic domain into an inactive conformation (Williams *et al.*, 1997a). These intramolecular interaction involve the inhibitory tyrosine binding to the SH2 domain, and this is then stabilised by the SH2-CD linker region associating with the SH3 domain (Gonfloni *et al.*, 1997). Interestingly, studies have shown that the SH3 interaction with the SH2-

CD linker is probably more important as the key regulator of the kinase domain because displacement of this interaction can lead to Src kinase activity regardless of the SH2 c-termini pTyr interaction (Moarefi *et al.*, 1997). This suggests that a possible mechanism for regulating Src kinase function is SH3 domain interactions. Certainly, the recently described PxxP-containing Unc119 protein has been shown to bind to Lck and Fyn via the SH3 domains leading to activation of these kinase, possibly by displacing the autoinhibitory interaction with the SH2-CD linker (Gorska *et al.*, 2004). However, it is still considered that phosphorylation of the C-termini Tyr is first required in initiating the SH3 SH2-CD linker interaction as deletion of Tyr 505 in Lck leads to a constitutively active kinase (Marth *et al.*, 1988).

Finally, Lck and Fyn may be regulated, in part by their subcellular localisation. As mentioned previously, features within the SH4 and also the unique regions are responsible for targeting these kinase to distinct areas within the cell. For example, s-acetylation of Lck and subsequent membrane localisation has been shown to be important to phosphorylation events in cell lines, as has Lck association with the co-receptors (Kabouridis *et al.*, 1997). In terms of Fyn localisation mirystoylation of Fyn within the unique region has been shown to mediate its association with the CD3 components (van't Hof and Resh, 1999), which may be essential to function. Fyn has also been suggested to interact with the mitotic spindle, but the significance of this interaction has not been established (Ley *et al.*, 1994). Furthermore, it has been suggested that Lck exists primarily outside lipid raft fractions in resting T cells,

whereas Fyn is found within these specialised domains, and that this may have functional significance (Filipp *et al.*, 2003).

1.6.3 Role of Src kinases in T cell development

Lck, and to a lesser extent Fyn, have both been shown to play important roles in the events regulating T cell development in the thymus. In the $Lck^{-/-}$ mouse, there is a profound reduction in thymus size due to a reduction in the number of DP cells. This is caused by a block at the DN3 to DN4 transition because of defective signalling for β-selection in the absence of Lck (Molina *et al.*, 1992). Those cells that are generated have lower CD5 levels and increased TCR expression due to the defective allelic exclusion of the TCRβ gene locus (Anderson *et al.*, 1993; Anderson *et al.*, 1992). The cells that mature in the $Lck^{-/-}$ thymus presumably do so through Fyn signals, as $Lck^{-/-}$ $Fyn^{-/-}$ cells are completely arrested at the DN3 stage (Groves *et al.*, 1996; van Oers *et al.*, 1996). Expression of LckY505F mutant molecules in Rag-$1^{-/-}$ that would normally be completely blocked at the DN3 stage can drive cells across the β selection checkpoint (Mombaerts *et al.*, 1994). Although the thymic development in $Fyn^{-/-}$ mice was reported to be grossly normal (Appleby *et al.*, 1992; Stein *et al.*, 1992). over expression of kinase-inactive Fyn was able to affect thymocyte signalling (Cooke *et al.*, 1991). Interestingly, the preferential role of Lck in β selection is not mediated by the kinase domain, as the expression of a chimeric molecule composed of the kinase domain of Lck fused to the SH4,SH3 and SH2 domains of Fyn was not able to restore thymic development in $Lck^{-/-}$ mice (Lin *et al.*, 2000).

In terms of regulating the transition from DP to SP thymocytes, Fyn may not play any role at this stage as protein levels were shown to be undetectable in DP thymocytes (Olszowy *et al.*, 1995). In contrast Lck has been heavily implicated in the signals that control lineage decision, partly because the impact of Lck deficiency on the SP population was even more profound than on the DP subset (Molina *et al.*, 1992). It has been shown that CD4 binds Lck mores strongly than CD8 and chimeric molecules composed of the extracellular portion of CD8 fused to the intracellular domain of CD4 were able to divert class I restricted TCR transgenic cells from the CD8 lineage to the CD4 lineage (Seong *et al.*, 1992). Moreover, decreasing Lck activity has been shown to promote CD8 differentiation (Legname *et al.*, 2000) and increasing activity sends cells to the CD4 lineage (Hernandez-Hoyos *et al.*, 2000). Furthermore, in our lab we have previously shown that co-ligation of DP thymocytes with bi-specific antibodies against either TCR/CD8 or TCR/CD4 always drove cells to the CD4 lineage, even when they expressed a class I-restricted TCR transgene (Bommhardt *et al.*, 1997). In contrast cross-linking the TCR/CD3 alone instructed cells to proceed to the CD8 lineage even in class II transgenic systems (Basson *et al.*, 1998). Although unexpected, stimulation methods involving the co-receptors would presumably lead to the activation of Lck, again suggesting that the strength of Lck signal helps to determine lineage fate. Collectively, these data suggest that lineage commitment does involve an element of instructive signalling and is not merely a stochastic event, as has been proposed.

1.6.4 Haematopoietic Src kinases in non- T cells

The BCR is composed of surface bound IgM molecules coupled to the invariant Igα and β chains, which each contain ITAM motifs. It is thought that the Src family kinase Lyn is responsible for phosphorylating these Tyr residues in order to initiate B cell signalling. Lyn is weakly associated with the non-phosphorylated ITAM in Igα, an interaction stabilised by its SH4 domain binding to the plasma membrane (Resh, 1994) . Furthermore, Lyn has been shown to phosphorylate both Syk and Btk, essential downstream mediators of BCR signalling (Kurosaki, 1999). Interestingly $Lyn^{-/-}$ B cells are hyper-responsive, and maybe due to the fact that Lyn has also been shown to phosphorylate the B cell inhibitory receptors FcγRIIB (Nishizumi *et al.*, 1998) and CD22 (Smith *et al.*, 1998). Thus Lyn may have a dual control over activation and signal termination (Xu *et al.*, 2005).

In terms of B cells signalling, Lck has recently been shown to be expressed in a subset termed B1a cells. The function of Lck in these cells seems to be suppressive as B1a cells are generally hyporesponsive to activation compared to other B cell subsets. However in the absence of Lck, the responsiveness of these B1a cells increase to resemble other B cell types (Dal Porto *et al.*, 2004). While Fyn has been shown to be expressed in B cells and associate with components of the BCR, there has been no clear function assigned in terms of BCR signalling, however $Fyn^{-/-}$ B cells were slightly hyporesponsive to IgD/IgM regulated activation, interestingly this was exacerbated when $Lyn^{-/-}$ $Fyn^{-/-}$ B cells were used (Horikawa *et al.*, 1999). Also Fyn has been implicated in the signalling of the CD38 expressed on B cells (Yasue *et*

al., 1997). Recently, Fyn expression has been reported in mast cells and has been shown to mediate a Lyn independent pathway of IgE mediated degranulation (Parravicini *et al.*, 2002). Interestingly, the loss of Lyn expression was able to render this Fyn dependent pathway hyper-responsive suggesting that Lyn may also play a negative regulatory role in Fyn-mediated degranulation (Parravicini *et al.*, 2002).

1.4.4 Molecules that modify Lck/Fyn activity

Having discussed the initiation of T cell signalling at the molecular level, and the importance of Lck and Fyn in these events, it is important to consider how the activity of these kinases are regulated in a T cell with respect to signal activation and termination. Over recent years a number of molecules have been described that interact with Lck/Fyn in such as way as to influence activity, either through changing the phosphorylation status of regulatory Tyr residues, or by altering the molecular conformation. These molecules are summarised in Figure 1.3.

1.6.6 Negative molecular regulators

Emerging evidence suggests that Lck and Fyn are regulated by a multiprotein complex containing a recently identified transmembrane adapter molecule termed PAG. PAG has been shown to reside in the GEM and is phosphorylated in resting T cells on Tyr317 facilitating an associate with Csk. Csk is a kinase that has been shown to phosphorylate the C-termini Tyr residue in Fyn and Lck promoting the closed conformation. Moreover, Csk has been shown to constitutively associate with

two phosphatases PEP and PTP-PEST (Davidson and Veillette, 2001), both of which are capable of removing the phosphate from the Tyr residues within the kinase domains of Lck and Fyn. Interestingly, Fyn has been implicated as the preferential kinase able to phosphorylate Tyr317 in PAG and facilitate Csk/PEP binding (Yasuda *et al.*, 2002). Furthermore, residual Fyn kinase activity can be detected in GEM from resting T cells suggesting that Fyn my function to keep T cells in a quiescent state. Interestingly the SH3 domain of Fyn has been shown to associate with a proline rich segment of PAG, and this may assist in keeping Fyn in a kinase active conformation, regardless of the phosphorylation status of the C-termini Tyr.

The function of Lck and Fyn may also be regulated by ubiquitin-mediated degradation. The E3 ubiquitin ligase c-Cbl has been shown to associate with Fyn and Lck (Feshchenko *et al.*, 1998; Fukazawa *et al.*, 1995). It may function to downregulate signalling in two ways. Firstly, it may tag them for lysosomal degradation, however c-Cbl may also serve to remove Lck and Fyn from the GEM (Hawash *et al.*, 2002). T cells from c-Cbl$^{-/-}$ mice are hyper-responsive to stimuli (Murphy *et al.*, 1998). Finally, there is also some suggestion that SHP-1 phosphatase may be able to dephosphorylate the kinase-promoting residue in Lck, and this may downregulate function (Stefanova *et al.*, 2003).

1.6.7 Positive molecular regulators

Recently a small molecule called Unc199 has been shown to positively regulate Lck/Fyn activity in T cells by virtue of its association with the SH3 domains of these

kinases (Gorska *et al.*, 2004). It is considered that binding of Unc199 to the SH3 domain destabilises the closed molecular conformation, allowing the kinase domain to be accessible. However this would presumably not alter the SH2, C-termini Tyr association or the phosphorylation status of this residue. It has also been proposed that dephosphorylation of the C-termini inhibitory Tyr residue is important to Lck/Fyn activity and that this may be mediated by the CD45 phosphatase, however, no study has conclusively shown that the phosphorylation status of the C-termini Tyr residues alters upon T cell activation (Hasegawa *et al.*, 2004). CD45 may simply function to dephosphorylate Tyr317 in PAG (Davidson *et al.*, 2003), however SHP-2 has also been implicated in this process (Zhang *et al.*, 2004). Loss of phospho-PAG Tyr 317 facilitates the loss of Csk/PEP from the GEM and eliminates the inhibition of Lck/Fyn function (Lindquist *et al.*, 2003). Interestingly, a recently identified molecule G3BP has been shown to sequester Csk from the GEM and may function to counter the role of PAG (Rahmouni *et al.*, 2005). Another recently identified adapter molecule termed LIME may also function as a positive regulator of Src kinase activation (Brdickova *et al.*, 2003; Hur *et al.*, 2003). Upon CD4 cross-linking, LIME is phosphorylated, presumably by Lck, and can then bind the SH2 domain of Lck and also Csk/PEP complexes. Due to the LIME-LckSH2 interaction, the fact that the C-termini Tyr is phosphorylated by Csk does not induce molecular closure, and thus LIME may be considered to positively regulate Lck activity. It has been proposed that the open Lck with Tyr505 phosphorylation may generate a binding site for other SH2 containing proteins (Brdickova *et al.*, 2003). This may include Fyn, as recent work has suggested that Lck may positively act on Fyn kinase activity (Filipp *et al.*,

2004; Filipp *et al.*, 2003). Finally, it has also been suggested that Lck can be phosphorylated directly by Erk on serine residue 59. This is thought to antagonise the proposed negative function of the phosphatase SHP-1 (Stefanova *et al.*, 2003).

1.7 Thesis aims

The overall aim of this thesis is to use transgenic mouse models to address what contribution Lck and Fyn make to T cell responses. Certainly, from the wealth of biochemical data being accumulated, it is possible that these kinases may not solely be responsible for driving T cell activation, but may also play a role in regulating the response.

In the first results chapter I will describe the basic characterisation of antigen-activated $Fyn^{-/-}$ CD8 T cells expressing a monoclonal class I-restricted TCR. The next two chapters detail the possible signalling mechanisms controlling the phenotype of the $Fyn^{-/-}$ CD8 T cells and the subsequent influence on effector function. The final results chapter describes the outcome of concomitantly eliminating Fyn expression and reducing Lck levels on the maintenance of peripheral T cell tolerance.

Figure 1.1: T cell signalling pathways and Lck / Fyn

The major T cell signalling pathways influencing gene expression that are thought to be downstream of the TCR / CD3 / CD4/8 receptor complex are shown. Also the contribution of CD28 and SLAM to signalling is also included. Molecules that are denoted with a red asterix have been implicated as being directly able to interact with Fyn and/or Lck.

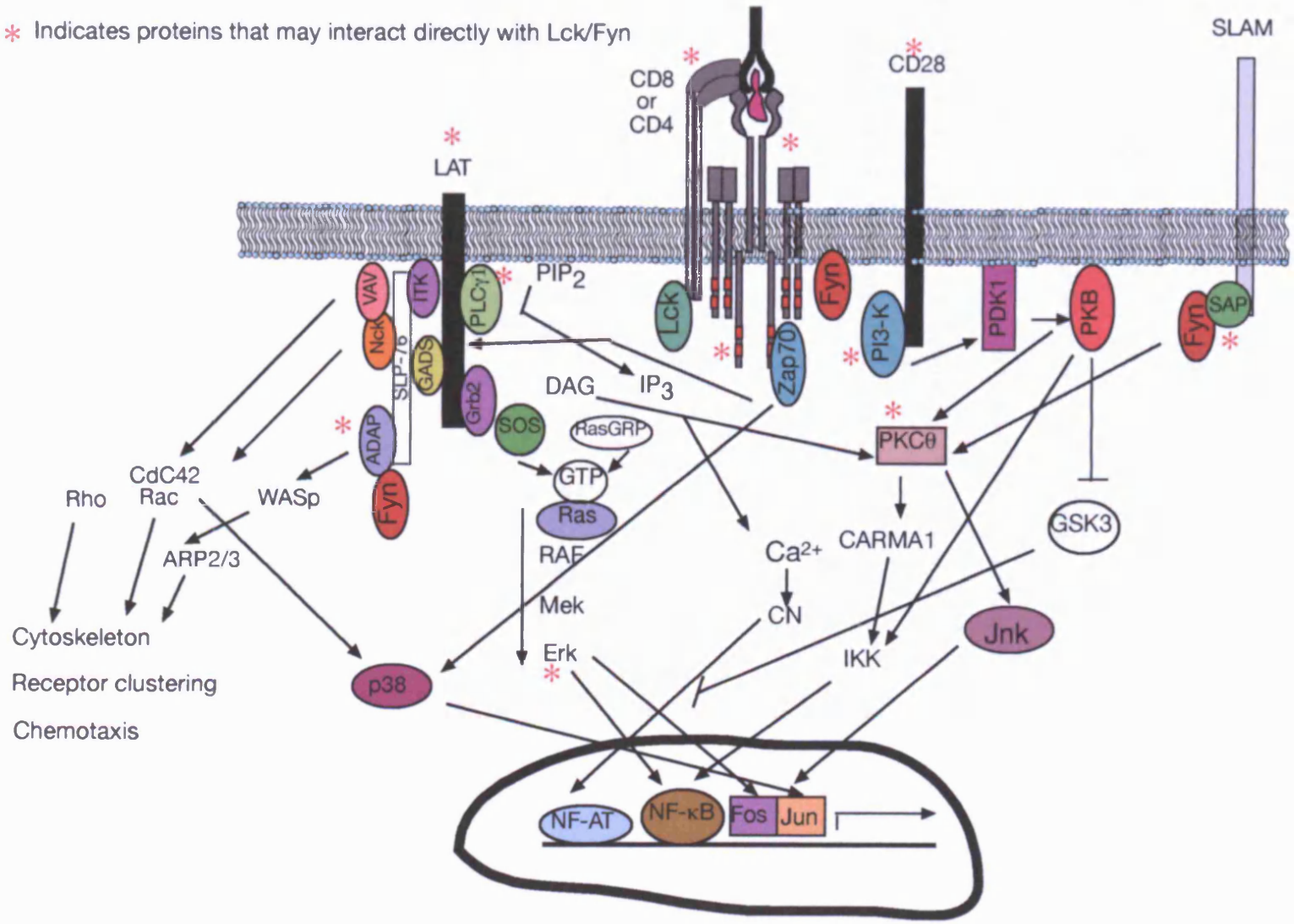
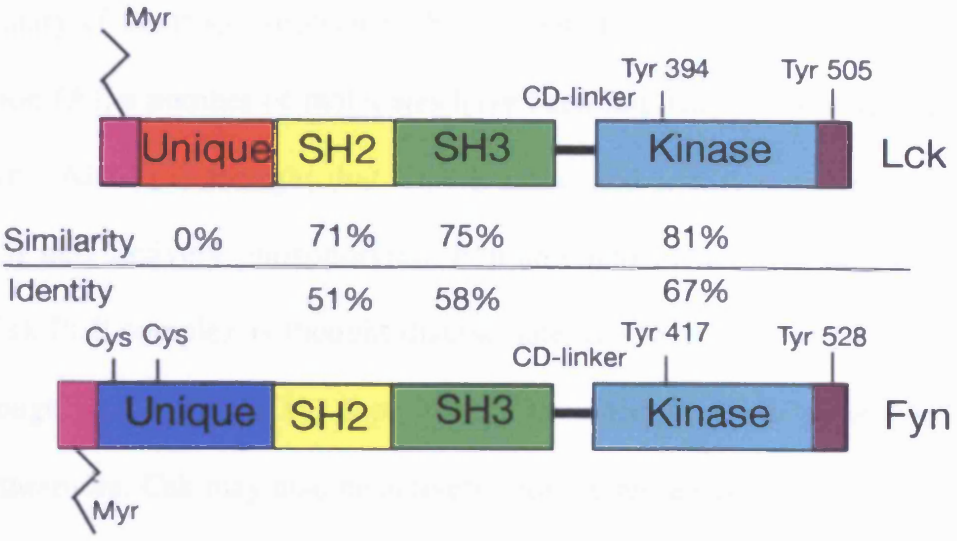

* Indicates proteins that may interact directly with Lck/Fyn
CD8
or
CD4
CD28
SLAM
LAT
PIP2
VAV
ITK
PLCγ1
Nck
SLP-76
GADS
Grb2
SOS
RasGRP
GTP
Ras
RAF
Mek
Erk
Lck
Fyn
Zap70
PI3-K
PDK1
PKB
SAP
DAG
IP3
PKCθ
ADAP
Fyn
CdC42
Rac
Rho
WASp
ARP2/3
Cytoskeleton
Receptor clustering
Chemotaxis
p38
Ca2+
CN
CARMA1
GSK3
IKK
Jnk
NF-AT
NF-κB
Fos
Jun

Figure 1.2: The structural feature of Src, Lck and Fyn

The arrangement of protein domains is shown for Lck and Fyn (A). Also the % of chemically similar amino acid residues is shown for each domain, as well as the % of identical residues. These were calculated using the pair-wise protein BLAST option on the NCBI website. The sequence accession numbers used were P39688 (Fyn) and P06239 (Lck). The crystal structure of inactive chicken Src (B) lacking the SH4 domain is shown (Williams *et al.*, 1997)

A


Myr
Tyr 394
Tyr 505
CD-linker
Unique
SH2
SH3
Kinase
Lck
Similarity 0% 71% 75% 81%
Identity 51% 58% 67%
Cys Cys
Tyr 417
Tyr 528
CD-linker
Unique
SH2
SH3
Kinase
Fyn
Myr

B

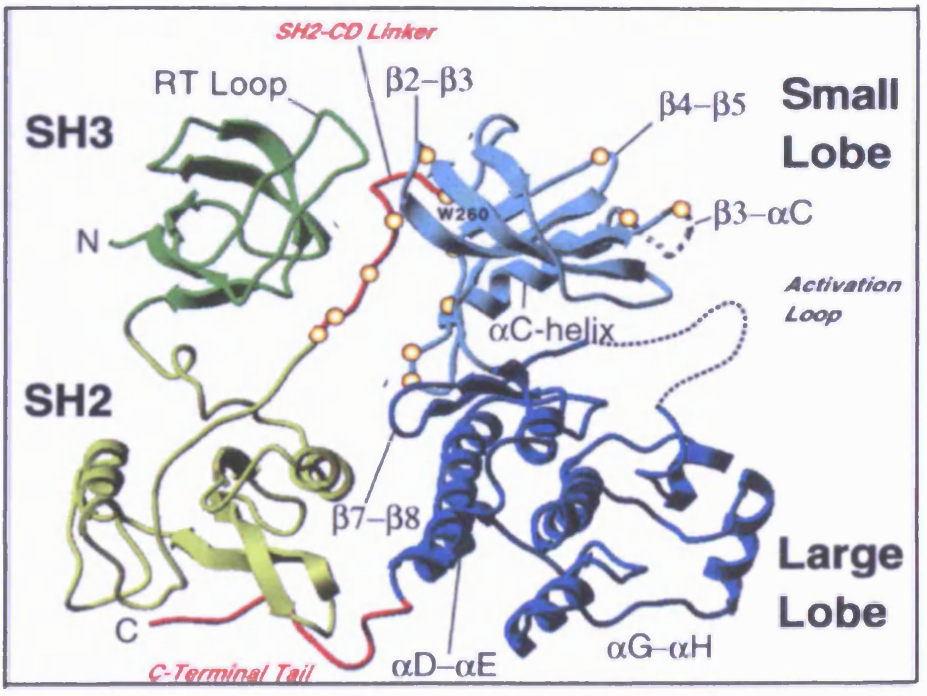
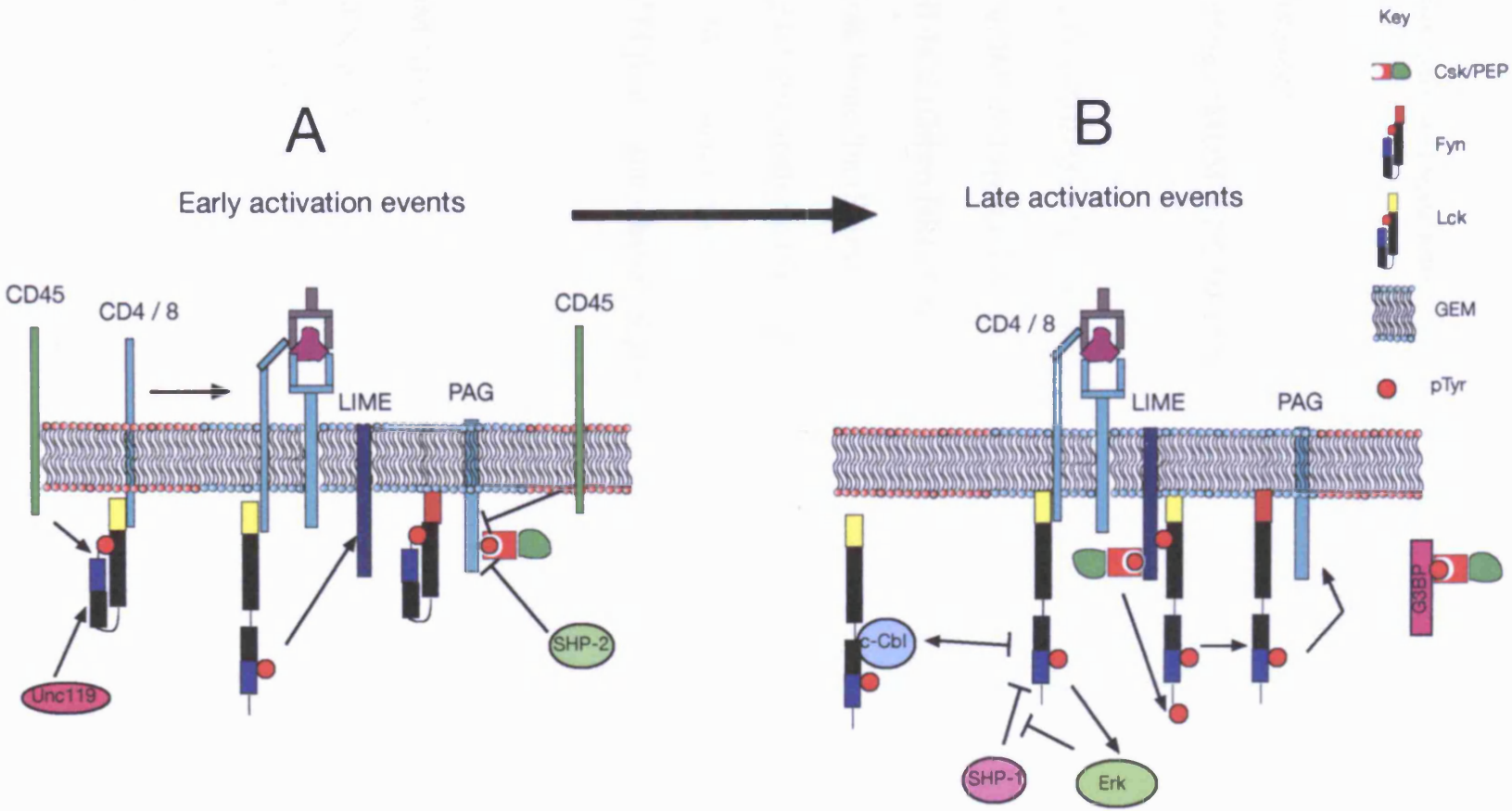

SH2-CD Linker
RT Loop
β2–β3
β4–β5
Small Lobe
SH3
N
W260
β3–αC
Activation Loop
αC-helix
SH2
β7–β8
Large Lobe
C
C-Terminal Tail
αD–αE
αG–αH

Figure 1.3: Molecular regulation of Lck and Fyn

A summary of the major molecules that regulate the activity of Lck and Fyn. Upon activation (A), a number of molecules have been implicated in the activation of Lck and Fyn. Also it is thought that CD8/4 associated Lck is brought into the GEM, where it may actively phosphorylate Fyn and also LIME. At the same time, the PAG/Csk/PEP complex is thought disassociate due to the loss of pTyr 317 of PAG. It is thought that PAG Tyr 317 dephosphorylation may be mediated by CD45 or SHP-2. Furthermore, Csk may also be actively sequestered away from the GEM by virtue of the newly identified phospho-protein, G3BP. Then at comparatively later stages of activation, Fyn may be responsible for rephosphorylating PAG Tyr 317 and mediate the reassembly of the PAG/Csk/PEP complex to downmodulate signals through Src kinases.


A
Early activation events
B
Late activation events
Key
Csk/PEP
Fyn
Lck
GEM
pTyr
CD45
CD4 / 8
CD45
LIME
PAG
Unc119
SHP-2
CD4 / 8
LIME
PAG
c-Cbl
SHP-1
Erk
G3BP

Chapter 2: Materials and Methods

2.1 Materials and solutions

Handling media

Air Buffered IMDM + 2% HI-FCS

RPMI 1640/ IMDM culture media

RPMI or IMDM (Sigma, UK)

10% HI-FCS (Gibco BRL, UK)

100 U/ml Penicillin (Sigma)

100 μg/ml Streptomycin (Sigma)

2 $x10^{-3}$ M L-Glutamine (Sigma)

5 $x10^{-5}$ M β-mercaptoethanol (Sigma)

ACK

150 mM NH_4Cl

10 mM $KHCO_3$

0.1 mM Na_2EDTA

FACS buffer

PBS + 0.5% Sodium Azide + 0.5% BSA

Dynal bead binding buffer

PBS (Gibco) + 2% HI-FCS

Bead wash buffer

PBS + 0.1% Tween-20 (Sigma)

Bead storage buffer

PBS + 40% Glycerol (BD chemicals, UK)

10x Cytokine permeabilisation buffer

1% NP40 (Sigma)

150 mM NaCl

50 mM Tris pH 7

Confocal perm buffer

PBS + 3% HI-FCS + 0.25% Triton-X 100 (Sigma)

2x Lysis buffer

300 mM Nacl

100 mM Tris pH.7

20 mM NaF

20 mM Di-sodium phosphate

2% n-Octyl-B-D-Glucopyranoside (Sigma)

0.2 mM Sodium Orthovanadate

10 ng / ml SPI (Antipain / Chymostatin / Leupeptin / Pepstatin all from Sigma)

2 μM PEFA block (Sigma)

3x reducing sample buffer

4% SDS

30% Glycerol

0.1875 M Tris pH 6.8

15% 2-Me

0.03% Bromophenol Blue

Lower gel buffer

0.4% SDS

1.5 M Tris-HCL pH 8.8

Upper gel buffer

0.4% SDS

0.5 M Tris-HCL pH 6.8

Running buffer

1X Tris / Glycine / SDS buffer (Bio-rad)

Transfer buffer

1X CAPS

Blocking buffer

PBS + 0.5% Tween-20 + 5% dry marvel milk

Wash buffer

PBS + 1% Tween-20

Stripping buffer

100 mM 2-Me
2% SDS
62.5 mM Tris pH 6.7
dH_20

2.2 Mice

Mice with a homozygous disruption of the *Fyn* gene were a gift from Dr P Soriano (University of Pensilvania) and have been described elsewhere (Stein *et al.*, 1992). Rag-$1^{-/-}$ mice (Mombaerts *et al.*, 1992) and F5 Rag-$1^{-/-}$ (Mamalaki *et al.*, 1992) have all been described previously. Rag-$1^{-/-}$ F5 $Fyn^{-/-}$ mice were generated by back-crossing F5 Rag-$1^{-/-}$ mice with $Fyn^{-/-}$ animals. The resulting F1 generation was inter-bred to homozygosity. $Lck^{-/-}$ mice harbouring the Lck-1 transgene controlled by the tetO/CMV promoter and $Lck^{-/-}$ mice containing the rtTA under the control of the huCD2 LCR have been described previously (Legname *et al.*, 2000) and Lck^{Ind} mice were generated by intercrossing these two lines. Lck^{Ind} $Fyn^{-/-}$ mice have also been described elsewhere (Seddon and Zamoyska, 2002a; Seddon and Zamoyska, 2002b). Lck expression was maintained by administering either 1 or 3 mg/g Dox food. The genotype of all mice was determined by a combination of PCR and FACS analysis of PBL. All mice were bred and housed under SPF conditions in concordance with home office regulations at the NIMR animal facility.

2.3 Cell isolation, purification and culture

2.3.1 Cell isolation protocols

2.3.2 Isolation of thymocytes and peripheral T cells

The cervical, axillary, inguinal and mesenteric lymph nodes were obtained from age matched groups of mice. In some cases the thymus and / or spleen were also removed (mice used for thymic analysis were always <6 weeks of age). Single cell suspensions were prepared by gently teasing the organs through a sterile gauze, allowing the cells to disperse into handling media. Cell were washed once, resuspended in handling media and counted using a Casy-1 automated cell counter (Scharfe system, Germany).

2.3.3 Generation of bone marrow-derived dendritic cells

The back legs (Tibia and Fibia) of C57BL/6 mice were obtained and the bone marrow was extracted using a fine gauge needle and 1 ml syringe to flush handling media into the bone. Bone marrow cells were washed once by centrifugation at 20°C and 10,000 rpm. Cell were then counted and resuspended in RPMI culture media. Then 5 x 10^6 bone marrow cells were cultured in 10ml of RPMI culture media containing 10% neat supernatant from the Ag8653 myeloma cell line that has been transfected with murine GM-CSF (20 U/ml). The cell were cultured at 37°C / 5% CO_2 in 9 cm petri-dishes (Nunc, Denmark) for an initial period of 4 days after which media was replaced to remove non-adherent granulocyte cells. At day 6-8 BMDC were harvested from the plates and were

either pulsed with antigen for 4 hrs at a density of 5 x 10^6 cells/ml in 50ml Falcon tubes at 37°C / 5% CO_2 or left unpulsed for control purposes. Antigen pulsed BMDC were washed three times before use.

2.3.4 Isolation of PBL

Briefly, ~100µl of blood was collected via the tail vein into 1.5ml eppendorf tubes containing 100µl of heparin (Sigma). Blood samples were then transferred to 6ml FACS tubes and 2ml of ACK was added and mixed briefly with vortexing. Samples were left for 10 minutes at room temperature, after which tubes were filled to the top with ice cold FACS buffer and spun at 1200rpm for 5 minutes at 4°C. The liquid was aspirated away to leave the pellet of RBC-depleted PBL.

2.3.5 Purification strategies

2.3.6 Dynal bead depletion

For some experiments it was necessary to negatively deplete to enrich for or purify the T cells. Briefly, cells were labelled on ice for 30 minutes with various biotinylated antibody cocktails (see table 2.1) at saturating concentrations in handling media (100 µl / 10^7 cells). Samples were then washed and resuspended in 4ml of Dynal bead binding buffer as it was essential that there was no biotin so as not to compete with the reaction. Dynal beads (M280, Dynal, Norway) were added at a ratio of 2:1, beads to cells for a further 30 minutes with constant rotation at 4°C. Cells bound to dynal beads (negative fraction) were then removed using a magnetic particle concentrator (Dynal). After a wash step cells

were resuspended in the appropriate media and counted. The success of the depletion was determined using flow cytometry.

Specificity	Clone	Conjugate	Supplier	Dilution
γδ receptor	GL-3	Biotin	BD	1/400
CD11b/Mac-1	M1/70	Biotin	BD	1/400
I-A^b	25-2-17	Biotin	BD	1/400
CD49d	DX-5	Biotin	BD	1/400
CD45R/B220	RA3-6B2	Biotin	BD	1/400

Table 2.1: Antibodies used for dynal bead depletion.

2.3.7 MACS and MOFLO sorting

An auto-MACS machine was used to obtain specific pure cell populations by positive selection when sterility was not important to the subsequent assay. Briefly, the desired cell population was first enriched for by removing unwanted cells using the Dynal bead strategy described previously (2.3.6). Then the remaining target population was labelled with a specific PE conjugated antibody for 30 minutes on ice at a density of 100 µl per 10^6 cells. After washing, cells were then incubated with 10µl per 10^6 cells of a 1:10 dilution of anti-PE MACS beads (Miltenyi biotech, UK) for 30 minutes on ice. Cells were then washed and passed through a 70 µM filter (BD Falcon, USA) to remove any cell clumps. Positive selection was achieved using the POSL programme for MACS selection based on the fact that the target population was often ≥10% of the total cell population. After separation, cells were counted for use in downstream applications.

For downstream application where sterility was essential, MOFLO separation was used. In this case unwanted cell types were also removed by Dynal beads (section 2.3.6). Cells were then labelled with fluorescently tagged antibodies against various cell markers such as CD4, CD8 and CD45RB for 30 minutes on ice. After washing cells were passed through a 70 μM filter and separation of cell populations was achieved using a MOFLO cell sorter (DAKO Cytomation, USA).

2.3.8 Pre-culture cell modifications

2.3.9 Cell labelling with intracellular fluorescent dyes

For experiments designed to measure proliferation, conjugate formation or in circumstances requiring the ability to distinguish two cell populations, cells were labelled with the CFSE or SNARF-1 (both Molecular probes, USA). Briefly, prior to labelling cells were washed once in serum free media to remove excess protein that could compete with CFSE/SNARF-1 binding to cellular proteins. Cells were then resuspended in Dulbeccos PBS (Gibco, UK) containing 0.1 μM CFSE or 1 μM SNARF-1 (prewarmed to 37°C) at a minimum density of 3×10^7 cells / ml for 10 minutes at room temperature. After labelling, cells were washed twice in handling media, counted and resuspended in the appropriate media for downstream applications.

2.4 In vitro T cell activation

2.4.1 Preparation of stimuli

2.4.2 Peptide

The F5 TCR transgenic line recognises the influenza virus nuclear protein NP68 (Ala, Ser, Asn, Glu, Asn, Met, Asp, Ala, Met) in the context of H-2D^b. This peptide was synthesised and purified in the NIMR division of Protein Structure.

2.4.3 Generation of microbeads

For some experiments, cells were stimulated using bead-immobilised antibodies. CELLection biotin binder beads (Dynal, Norway) were washed three times by adding 1 ml of bead wash buffer, placing the eppendorf into a MPC, and aspirating the liquid away. Beads were then resuspended in 500 µl of bead wash buffer containing 20 µl (10 µg) of each biotinylated antibody was added (see table 2.2). To ensure that the final concentration of antibodies on beads with anti-CD3/CD28 was comparable to beads with three different antibodies, an irrelevant IgG biotinylated antibody was also included on the CD3/28 beads to offer competition for biotin binding sites. Tubes were placed on a rotator for 30 minutes at RT after which the beads were washed 3 times in bead wash buffer as mentioned previously. Finally, beads were resuspended at an appropriate concentration in sterile bead storage buffer, and stored at -20°C till use. Before using beads in an assay they were washed once in culture media.

Specificity	Clone	Conjugate	Supplier
CD3ε	145-2C11	Biotin	BD
CD4	RM4-5	Biotin	BD
CD8β	53-5.8	Biotin	BD
CD28	37.51	Biotin	BD
IgG1	A85-1	Biotin	BD

Table 2.2: Antibodies used for microbead coating

2.4.4 pMHC dimer preparation

Peptide loaded recombinant soluble dimeric mouse MHC class I : Ig fusion proteins (BD Biosciences, UK, Cat no: 551323) were prepared as outlined in manufacturers instructions (hereon referred to as pMHC dimers). Briefly, the correct ratio of MHC dimer to peptide was calculated using the following equation:

$$Mp = \frac{MD^b \times R \times Dp}{DD^b}$$

Dp = Molecular weight of peptide (eg 8mer = 130 x 8 = 1,040 Daltons).

DD^b = Molecular weight of $H2D^b$: Ig = 250,000 Daltons.

R = desired excess molar ratio of 160

Mp = micrograms of peptide of interest

MD = micrograms of $H2D^b$: Ig in the reaction

The volume of the solution was adjusted to the desired concentration using PBS. Loading and assembly of the MHC dimer and peptide was achieved by an

overnight incubation at 37°C. In all experiments correct assembly was assessed by incubating loaded dimer with 1 x 10^6 F5 lymph node cells for one hour, then incubating with a PE labelled anti mouse IgG1 (BD) to visualise pMHC dimer molecules bound to F5 CD8 T cells.

2.4.5 Stimulation conditions

2.4.6 Soluble NP68

For antigen specific stimulation of F5 CD8+ T cells using soluble NP68 peptide and APC, preparations of total LN cells were seeded in a 96 well round bottom tissue culture plate (Nunc, Denmark) at a density of 1 – 2 x 10^5 cells per well in IMDM culture media. Samples were then incubated with a titration or a fixed dose of NP68 peptide at 37°C / 5% CO_2. Cultures were terminated at specific time points for analysis. In some experiments, cells were pre-incubated with 10 μg / ml CTLA4-Ig (gift from Peter Lane, University of Birmingham, UK) or control Ig for 45 minutes at 37°C / 5% CO_2. Then NP68 was added to begin the stimulation. Furthermore, in other specific experiments, cells were co-incubated with NP68 and either the anti-IL-2 antibody S4B6 (1:50 of dilution of the cell line supernatant), or 10 units of recombinant IL-2 (R&D systems, UK) or a 1/50 dilution of supernatant from the cell line X63-IL-2 for the full period of the culture.

2.4.7 Activation with beads

For stimulations using synthetic beads, F5 lymph node cell suspensions were prepared as outlined and were depleted of APC using dynal beads and labelled with CFSE (as described). Cell were plated out at a density of 2 x 10^5 cells per

well and the specified beads (see 2.4.3) were added at a final ratio of 1:1 cells to beads. Cells were then placed at 37°C / 5% CO_2 for the indicated time points.

2.4.8 pMHC dimer activation

A 96 well flat-bottomed plate (Nunc, Denmark) was coated overnight with a 5 μg/ml solution of NP68 loaded pMHC dimer solution prepared as outlined in section 2.4.4. LN cells from F5 mice were harvested and depleted for APC with anti-CD11b/I-A^b biotinylated antibodies using the strategy outlined in section 2.3.6. Cells were then spun down at 400rpm for 30 seconds onto the layer of pMHC dimers at a density of 2 x 10^5 cells per well and incubated for specific times. Negative control stimulations were carried out using pMHC-dimers loaded with the non-stimulatory GAG peptide.

2.4.9 Assaying cell function

2.4.10 4 – colour FACS staining and analysis

The expression of cell surface markers by *ex vivo* and *in vitro* activated cells was assessed using 4-colour flow cytometry. Briefly 1 x 10^6 cells were incubated with 100 μl of FACS buffer containing a 1/50 dilution of the anti-Fc receptor antibody 2.4G2 to reduce non-specific binding and specified dilutions of directly conjugated fluorescent antibodies or biotinylated antibodies against surface receptors (see table 2.3). Samples were incubated for 30 minutes on ice, followed by a wash step. If required, cells were incubated for 10 minutes on ice with a second layer streptavidin PerCP conjugated fluorophore (BD) to reveal the presence of biotinylated antibodies. Samples were again washed once and

resuspended in 100 µl of ice cold before acquisition on a FACScalibur machine (BD). Data analysis was carried out using the FloJo software analysis platform (Tree star, USA).

Specificity	Clone	Conjugate	Supplier	Dilution
Fc receptors	2.4.62	Un-conjugated	From cell line	1/50
CD45RB	16A	FITC	BD	1/400
CD25	7D4	FITC/APC	BD	1/200
TCRβ	H57-597	FITC/PE/APC	BD	1/400
CD8β	53-5.8	FITC	BD	1/400
CD5	53-7.3	FITC/PE	BD	1/400
Isotype cont.	XMG1.2	FITC	BD	1/200
CD4	GK1.5	PE	BD	1/400
CD8α	53-6.7	PE/APC/PerCP	BD	1/400 1/200
CD25	PC61	PE	BD	1/400
CD69	H1.2F3	PE/Biotin	BD	1/400 1/200
CD44	IM7	PE/APC	BD / Leinco	1/400 1/200
CD45RB	23G2	PE	BD	1/400
Isotype	R3-34	PE	BD	1/200

CD4	RM4-5	APC/PerCP	BD	1/400 1/200
Vβ4	KT4	Biotin	BD	1/50
Vβ5	MR9-4	Biotin	BD	1/50
Vβ6	RR4-7	Biotin	BD	1/50
Vβ7	TR310	Biotin	Caltag	1/50
Vβ8.1/2	MR5-2	Biotin	Caltag	1/50
Vβ8.3	1B3.3	Biotin	BD	1/50
Vβ10	B21.5	Biotin	Caltag	1/50
Vβ11	RR3-15	PE	Caltag	1/50
Vβ12	MR11-1	Biotin	Caltag	1/50
Vβ14	14-2	Biotin	BD	1/50
Vβ17	KJ23	Biotin	BD	1/50

Table 2.3: FACS antibodies used in this study

2.4.11 CFSE proliferation and calculations

For the measurement of cell proliferation, F5 cells were labelled with CFSE as described. After activation using any of the described protocols, samples were stained with the required antibodies combinations for the discrimination of cell

populations and activation marker expression. These were labelled with fluorophores that did not fluoresce in the FL-1 channel. Samples were then acquired on the FACScalibur and a minimum of 40,000 events was collected per sample in order to achieve good CFSE peak discrimination. Data files were analysed using CellQuest software and gates were set manually to determine cell frequencies within each round of division. The calculations used to analyse the proliferation were as follows. Data of the frequency of CFSE labelled cells (F) that had undergone division rounds (d) were used to calculate the adjusted frequencies (AdF), this was achieved by dividing F by 2^d. The burst size was obtained from the calculation $\Sigma(AdF \times d) / \Sigma F_{(for\ d > 0)}$. The percentage of cells triggered to divide was calculated by using the equation $(1 - [AdF_0 / \Sigma AdF]) \times 100$.

2.4.12 Intracellular cytokine / granzyme staining

For the detection of cytokines by intra-cellular cytokine staining, antigen activated cells or *ex vivo* cells were treated with final concentrations of 0.5 μg/ml PdbU, Ionomycin (Sigma) and 1 μg/ml Brefeldin A (Sigma) for 4 hours at 37°C 5% CO_2. Cells were then washed and stained for the expression of surface markers (as outlined). After a wash step, cells were resuspended in 100 μl of PBS and 100 μl of 6% paraformaldehyde was added to give a final concentration of 3% fixative. After 20 minutes on ice, cells were washed and resuspended in 100 μl of 1x cytokine permeabilisation buffer for exactly 3 minutes after which 100 μl of FACS buffer was added per well. Cells were washed once and resuspended in 100 μl of FACS buffer containing fluorescently labelled anti-cytokine antibodies or irrelevant isotype controls (see table 2.4). After a minimum 30 minute

incubation on ice, cell were washed once and acquired on a FACScalibur (BD). For granzyme staining, no PdbU, ionomycin or Brefeldin was used prior to intra-cellular staining.

Specificity	Clone	Conjugate	Supplier	Dilution
IFNγ	XMG1.2	FITC/PE	BD	1/200
IL-2	S4B6	PE	BD	1/200
IL-2	JES6-5H4	APC	BD	1/100
Granzyme B	GB12	PE	Caltag	1/100
Isotype	MOPC-21	PE	Caltag	1/100

Table 2.4: FACS antibodies for intracellular staining

2.4.13 CTLL-2 assay

In certain circumstances, media was harvested from activated cells and the IL-2 content was measured using the CTLL-2 bioassay. Briefly, 50 µl of supernatant from stimulated F5 cells were harvested at the defined time points. And incubated with a total of 5 x10^3 CTLL-2 cells (ATCC cat no. TIB 214) for 24 hours at 37°C / 5% CO_2. At the same time CTLL-2 were incubated with a titration of recombinant IL-2 to generate a standard curve. After 24 hours, 10 µl of Almar blue solution (Biosource, UK) was added to each well and incubated over night. The plate was then read using a Luminescence Spectrometer LS50B (Perkin Elmer, UK) at 590 nm and data analysed using FL WinLab software (Perkin Elmer). The standard curve was used to calculate the IL-2 content in U/ml.

2.4.14 IL-2 capture assay

Detection of IL-2 production using the commercially available IL-2 capture assay (Miltenyi Biotech, UK) was carried out as outlined in the manufactures protocol. Briefly, a minimum of 1 x 10^6 activated cells were washed once, placed in a 1.5 ml sterile eppendorf tube and labelled with 100 µl of a 1/10 dilution of IL-2 capture antibody in ice cold IMDM for 10 minutes on ice. Volumes were then made up to 1 ml with pre-warmed culture media and placed at 37°C / 5% CO_2 for 1 hour with constant agitation. After this cells were washed once and labelled with 100 µl of ice-cold media containing a 1/10 dilution of the anti-IL-2 PE detection antibody and any other surface marker antibodies required. A 1/100 dilution of the anti-Fc receptor antibody 2.4G2 was also added at this stage. Cells were incubated for 15 minutes on ice, washed once and acquired immediately on a FACScalibur.

2.4.15 Conjugate assay

For the detection of T cell-APC conjugate formation by FACS, BMDC were grown 7 days prior to the assay as described. The F5 T cells were labelled with CFSE and the BMDC with SNARF-1, also as outlined previously. To induce conjugates, cells suspended in IMDM culture media were placed in 1.5ml eppendorf tubes, mixed at a 1:1 ratio (5 x 10^5 per cell type) and spun down at 1200rpm for 10 seconds at room temperature. The pellets were quickly and vigorously resuspended and tubes were placed at 37°C / 5% CO_2 for 10 minutes, after which they were acquired on the FACScalibur. For controls, gates were set

using T cells or BMDC alone or with T cells conjugated with non-peptide-pulsed APC. Analysis was carried out using CellQuest software.

2.4.16 CTL assay

Prior to the assay, the target EL-4 thymoma line was maintained in IMDM culture media and split at ~80% confluence so cells were in the log phase of growth the day before the assay. Three days prior to the assay, suspensions of LN and spleen were prepared from F5 mice and activated with 1 μM NP68 at a cell density of 5 x 10^5 cell/ml in culture IMDM. Cultures were grown in upright culture flasks and with a minimum of 10 ml cell suspension for 3 days at 37°C / 5%CO_2. On the day of the assay F5 cells and EL-4 cells were washed once and counted. The EL-4 cells were then resuspended at 6 x 10^6 per ml in serum free AB IMDM and split into two equal groups, one aliquot was pulsed with 10 μM of NP68 and the other was left un-pulsed. EL-4 cells were then incubated for 3 hours at 37°C / 5%CO_2. After a wash step, EL-4 cells were labelled with 1.85 Mbq of ^{51}Cr and incubated for 1.5hrs at 37°C / 5%CO_2. Effector cells were added at a concentration representing the top effector to target ration and serially diluted 1/3 across the 96 well round bottom plate. Then a total of 1 x10^4 pulsed, ^{51}Cr labelled EL-4 cells was added to respective wells to give the correct effector to target ratios in the well. The maximum ^{51}Cr release was measured by lysing pulsed targets with 1% Triton-X. The plate was spun down at 1000 rpm for 1 minute then incubated at 37°C / 5%CO_2 for 4 hours. After this time 25 μl of supernatant was harvested using a micro-96 harvester (Skatron instruments, USA) and transferred to a printed filter mat A (Wallac, Finland). These mats were dried in the microwave

for 1 minute, and immersed in liquid scintillant (Wallac). The degree of ^{51}Cr release was measured using a 1205 automated β-plate counter (Wallac). The NP68 specific ^{51}Cr release was calculated using the following equation.

$$\frac{\text{(Experimental - Spontaneous release)}}{\text{(Maximal release – Spontaneous release)}} \times 100 = \%\ \text{specific}\ ^{51}Cr\ \text{release}$$

2.4.17 Intracellular Ca^{2+} fluxing

For antigenic stimulation BMDC were grown for 1 week prior to the experiment as outlined. On the day of the experiment these were either pulsed for 4 hrs with 10 μM NP68 or with media alone at 37°C / 5% CO_2. Suspensions of F5 CD8 T cells were prepared as described and loaded with 4 μg/ml of Indo-1 AM (Molecular probes) in RPMI 1640 media + 1% BSA at a density of 2×10^6 cells per ml by incubating in a 37°C water bath for 40 minutes followed by a wash step. For antibody induced calcium fluxing samples were incubated with saturating concentrations of either biotinylated 2C11 (BD), biotinylated CD8β (BD) or both for 15 minutes on ice, followed by a wash step. Calcium flux measurements were carried out by monitoring the change in FL-4 / FL-5 fluorescence ratio using a LSR flow cytometer (BD). In the case of antibody induced calcium flux, samples were acquired for 30 seconds to establish a baseline, then 20 μg avidin was added in order to cross link the pre-bound biotinylated antibodies. Samples were then returned to the LSR and acquired for a further 370 seconds. To measure antigen-specific calcium fluxes T cells were acquired for 30 seconds to establish a baseline. Cells were mixed 1:1 with either NP68 pulsed DC's or with DC's alone and centrifuged at 12,000 rpm for 10 seconds to induce conjugate formation.

After resuspension, samples were then returned to the LSR and acquired for a further 370 seconds.

2.4.18 Real-time PCR analysis

Purified F5 cells were obtained from the LN of mice and 1 x 10^6 cells were activated on a layer of NP68 pMHC dimers. At specific time points, cells were harvested and spun down at 1200rpm at 4°C for 1 minute. Media was aspirated away from the pellet and this was resuspended in 1 ml of Trizol (Invitrogen, UK). RNA was extracted using RNeasy Kit (Qiagen, Germany) and reverse-transcribed with oligo (dT) 16 (Applied Biosystems, USA) according to the manufacturer's protocol. cDNA served as template for the amplification of specific genes and the housekeeping gene Hprt transcripts by real-time PCR using Assays-on-Demand gene expression probes (Applied Biosystems, USA), Universal PCR Master Mix (Applied Biosystems, UK) and the ABI-PRISM 7000 Sequence detection system (Applied Biosystems, USA). Target gene expression was calculated using the comparative Ct method for relative quantitation upon normalization to Hprt gene expression.

Gene	Cat no.	Supplier
Hprt	Mm00446968	Applied Biosystems
IL-2	Mm00434256	Applied Biosystems
IFNγ	Mm00801778	Applied Biosystems
c-Rel	Mm00485657	Applied Biosystems
c-Jun	Mm00495062	Applied Biosystems
JunB	Mm00492781	Applied Biosystems

c-Fos	Mm00487425	Applied Biosystems
FosB	Mm00500401	Applied Biosystems
NF-ATc1	Mm00479445	Applied Biosystems

Table 2.5: Real time PCR primers used

2.4.19 Confocal analysis

A day prior to the experiment, 12 mm diameter round cover-slips were placed into a 24 well plate (Nunc). Coating of the coverslips was achieved by the addition of 500 µl of 0.01% poly-l-lysine solution (Sigma) for 3 hours at room temperature. The solution was aspirated away and the slips were left to air-dry overnight. Cell suspensions from F5 mice were prepared and activated with soluble NP68 as described. After 1-4 hours, cells were washed in PBS and resuspended at a density of 3 x 10^6 cells per ml. A total of 100 µl of cell suspension was then added slowly to the centre of the poly-l-lysine coated cover-slips. These were incubated for 10 minutes at room temperature after which the 100 µl was carefully removed from each slip. Next 100 µl of 2% paraformaldehyde was added carefully to each slide and incubated for 15 minutes at room temperature. After this the paraformaldehyde was removed and slides were washed 3 times using 500 µl of PBS containing 3% FCS. Next, 500 µl of confocal perm buffer was added for 10 minutes to permeabilise cells for intra-cellular staining. Cells were then incubated with a 1/20 dilution of rabbit-anti mouse c-Rel antibody (Santa cruz, USA) or an irrelevant rabbit Ig isotype control, made up in the confocal perm buffer, or solution alone, for 45 minutes at room temperature. After this samples were washed 3 times in confocal perm buffer and incubated with a 1/50 dilution of anti-rabbit Ig $F(ab')_2$ fragments conjugated to Alexa 440 (Molecular probes) or

buffer alone for 30 minutes at room temperature. Cells were washed 3 times in 500 µl of confocal perm buffer and 100 µl of 2% paraformaldehyde was added for 15 minutes. Finally slips were washed 3 times with 500 µl of PBS alone and mounted on slides with one drop of I mounting media containing PI (Vecta-shield, USA). Samples were then acquired using a Leica delta vision confocal microscope (Leica, USA).

2.4.20 Duration of stimulation experiment

Prior to the assay, 96 well flat-bottom plates (Nunc) were coated with 5 µg/ml of NP68 pMHC dimers overnight and washed 10 times in PBS on the day of the assay to remove residual peptide. Suspensions of F5 cells were prepared and depleted of all APC using dynal beads as described, and a total of 5 $x10^5$ T cells were spun down onto the activating layer then incubated at 37°C / 5% CO_2. At specified time points, cells were taken off the dimer layer, washed 5 times and transferred to a fresh 96 well flat bottomed plates. To control for any carrier over of antigen, 1 x 10^5 CFSE labelled naive F5 cells were spiked into the wells. Also to minimise further activation 10 µg/ml of the anti-MHC class I blocking antibody (28-14-8 anti- mouse H-$2D^b$, BD) was also added to the wells. The plate was cultured at 37°C / 5%CO_2 and was analysed after 24 hours for the expression of activation markers and the detection of intra-cellular cytokines as described previously.

2.4.21 Inhibition of Erk or Src activity

For the measurement of IL-2 after treatment with the Mek inhibitor PD90859 (Calbiochem, USA), F5 cells were purified as described from the LN of mice and activated on a NP68 pMHC dimer layer. Cells were treated with 100 µl of 20 µM PD90859 in culture media per 1 x 10^6 cells, either for 30 minutes prior to activation on the layer or for the specified time points after which the inhibitor was removed by washing cells 3 times at 1000 rpm and resuspending in fresh IMDM culture medium and returning to the stimulus. IL-2 production was measured after a total of 24 hours in culture by intra-cellular cytokine staining as described. For the inhibition of Src kinases, either 20 µM PP2 or PP3 (Both Calbiochem) was added to cells, either 30 minutes before adding stimulus, or after 3 hours of activation. IL-2 analysis was conducted as described for PD90859 experiments.

2.5 Measurement of *in vivo* T cell function

2.5.1 In vivo F5 activation with A/NT/60-68 flu virus

Cells from F5 mice were isolated as described and 3 x 10^6 cells suspended in 150 µl of handling media with no serum were injected via the tail vein into Rag-$1^{-/-}$ mice along with an optimal dose (PfU) of the A/NT/60-68 influenza virus. After 7 days, recipient mice were sacrificed and LN and spleen were collected and analysed for cell number, cell phenotype and intra-cellular cytokine production by

FACS. As controls, F5 cells were transferred without virus. Recipients were routinely housed in SPF conditions at the NIMR animal facility.

2.5.2 Transfer of cells into Rag-1$^{-/-}$ hosts

Specified cell populations were isolated using both negative and positive selection strategies outlined previously. When the desired purity was achieved and checked by FACS, defined numbers of cells were injected into Rag-1$^{-/-}$ hosts via the tail vein. The success of transfer was assessed by bleeding mice after 24 hours and looking for the frequency of PBL by FACS. Recipients were then housed in SPF conditions at the NIMR animal facility. Mouse weights were monitored on a weekly basis.

2.5.3 Analysis of GI tract pathology

The entire GI tract was removed from mice and flushed with 20 ml of PBS. Then the GI tract was flushed with Bouins fixative and stored in the dark at room temperature, submerged in Bouins solution. For histology, portions were cut with a scalpol and embedded in wax and sections and stained with H & E at the NIMR division of histology. Slides were then analysed on a standard light microscope with digital camera attached (Fuji). Photos were taken using 100X magnification and 2X digital zoom.

2.6 Biochemical analysis

2.6.1 Stimulation conditions

For stimulation of F5 cells for biochemistry, a minimum of 5 x 10^6 cells in culture IMDM were activated on NP68 pMHC dimer layers for defined time points at 37°C / 5% CO_2. After these periods cells were removed from the layer and 10 µl were kept for FACS analysis of activation markers and the rest were transferred to pre-chilled 1.5 ml eppendorf tubes and spun down at 1000 rpm, 4°C for 30 seconds to pellet cells. After the supernatant was aspirated away, pellets were snap frozen in liquid nitrogen and stored at -70°C till use.

2.6.2 Sample preparations

2.6.3 Cell lysis

Cell pellets were lysed in a minimum of 500 µl of ice-cold 1x lysis buffer by repeat pipetting. Tubes were then left on ice for 20 minutes after which samples were spun down at 2000 rpm for 10 minutes at 4°C. The lysate was then transferred to clean pre-chilled 1.5 ml Eppendorf tubes.

2.6.4 Acetone precipitation

To concentrate the protein content of the lysates, 1.5 volumes of ice-cold acetone was added to each tube containing the lysate. Tubes were mixed and left

overnight at 4°C. Subsequently, acetone precipitates were washed 3 times in 70% ethanol always at 4°C, after which pellets were resuspended in the desired volume of 3x reducing sample buffer and placed on a mechanical tube shaker at 4°C for 2-3 hours. Samples were then either boiled for 5 minutes and loaded directly onto the SDS-PAGE gel or snap frozen and stored at -70°C.

2.6.5 Gel preparation, running and transfer

2.6.6 Preparation of lower gel and upper gel sections

Briefly, the plates were washed in water and 100% ethanol and dried thoroughly. After assembling the plates with 1mm spacers (Bio-rad), typically a 10% PAGE gel was used and made as follows:

7.5 ml Lower gel buffer
10 ml of 30% w/v Acrylamide stock (Protogel, National Diagnostics, UK)
100 µl of 10% APS (Bio-rad)
10 µl of TEMED (Bio-rad)
dH_20 to 50 ml

The gel was poured and a layer of H_2O saturated Butanol was placed onto the top. The gel was left for 1-2 hours to polymerise. The top of the lower gel section was washed with an excess of DI H_2O and the upper gel was made up as outlined:

2.5 ml Upper gel buffer
1.5 ml of 30% w/v Acrylamide stock

6 ml dH_20

50 µl of 10% APS

10 µl of TEMED

After pouring the upper gel, the 1mm well former was added and the gel was left to polymerise for ~1hr. After this time, all wells were washed with 1x running buffer.

2.6.7 Loading and running of gels

Prior to loading on the gel, samples were boiled for 5 minutes in a heat block at 100°C. After a brief period of centrifugation (for ~5 seconds), samples were loaded onto the gel with the molecular weight markers (Rainbow markers, Bio-rad, USA). Gels were typically run at 25 mA for 4-5 hours, or at 5 mA overnight using the Hoefer SE400 gel system (Amersham Biosciences, USA) with running buffer in the upper and lower chambers and a p5500XT DC power pack (Hoefer Scientific Instruments, USA).

2.6.8 Transfer conditions

For transfer of protein from the SDS-PAGE gel to a nitrocellulose membrane the following protocol was used. The membrane (Immobilon P, Millipore, USA) was activated by a brief submersion in methanol. The transfer was set up with the cassettes, sponges, gel and membrane all submerged in transfer buffer. The order

of assembly from the cathode to anode was, sponge, 2x filter paper, gel, membrane, 2x filter paper, sponge. Transfers were conducted using the Trans-Blot Cell system (Bio-rad). For 4 hour transfers the condition were 100 v 400 mA and for overnight transfers the condition were 30 v 100mA.

2.6.9 Western blotting analysis

2.6.10 Antibody incubations

Membranes were blocked in blocking buffer for a minimum of 2 hours at room temperature with constant agitation. Membranes were then incubated with blocking buffer containing the appropriate dilution of antibody for the optimal time period (see table).

Specificity	Residues	Supplier	Dilution	Incubation
pErk1/2	Tyr	Biosource Int	1/1000	4 hours
Total Erk 2	N/A	Cell signalling	1/2000	Overnight
pJnk1/2	Thr/Tyr 165	Cell signalling	1/2000	Overnight
Total Jnk1/2	N/A	Cell signalling	1/2000	Overnight
anti-Rabbit HRP	N/A	Cell signalling	1/3000	1 hour

Table 2.6: Antibodies used for western blotting

of assembly from the cathode to anode was, sponge, 2x filter paper, gel, membrane, 2x filter paper, sponge. Transfers were conducted using the Trans-Blot Cell system (Bio-rad). For 4 hour transfers the condition were 100 v 400 mA and for overnight transfers the condition were 30 v 100mA.

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pJnk1/2	Thr/Tyr 165	Cell signalling	1/2000	Overnight
Total Jnk1/2	N/A	Cell signalling	1/2000	Overnight
anti-Rabbit HRP	N/A	Cell signalling	1/3000	1 hour

Table 2.6: Antibodies used for western blotting

After this point, the membrane was submerged in wash buffer for a minimum of 1 hr with at least 4 changes of buffer. Membranes were then incubated with the appropriate HRP conjugated secondary antibody in blocking buffer (see table 5.2) for 1 hr after which washing was carried out as before. For chemiluminescent visualisation of bands, the membrane was covered with ECL solution (Amersham, UK) for 1 minute with agitation and placed in a screened cassette. Membranes were then exposed to blue film (Kodak MXB Film, France) for the desired period in darkness and developed using a FPM 2100 X-ray film processor (Fuji). For the detection of total protein for loading controls, membranes were stripped using stripping buffer for 20 minutes at 60°C then the process was repeated again using antibodies against total protein.

2.6.11 Quantification

The measurement of band intensities relative to loading controls was carried out using a Calibrated imaging densitometer (GS-710, Bio-rad). Briefly, films were scanned and the density of band signal was measured. The densitometry values generated for the phospho-bands were divided by the values then obtained for the loading controls and the ratio of phospho to total protein was calculated. For replicate experiments, the corrected ratios obtained for WT cells were set at 100% for each time point analysed. The mutant was then expressed as a percentage of these values at each respective time point.

Chapter 3: The role of Fyn in peripheral CD8 T cell activation

3.1 Introduction

Fyn deficient mice have been previously generated by two independent groups and the characterisation of the thymocytes and peripheral T cells have been described (Appleby *et al.*, 1992; Stein *et al.*, 1992). Soriano and colleagues used a knockout strategy that eliminated expression of both Fyn splice variants (FynT and FynB) by disrupting exon 2 that contains the initiator methionine (Stein *et al.*, 1992). In contrast Perlmutter and co-workers used a strategy that only disrupted exon 7B, which is incorporated into FynT transcripts and therefore did not affect FynB expression (Appleby *et al.*, 1992). When analysing thymic development, both groups found that it was unaffected. Appleby et al showed that the thymic profiles of $Fyn^{-/-}$ mice were comparable to WT mice and did not exhibit the profound block at the DN3 to DN4 transition evident in thymi from $Lck^{-/-}$ animals (Molina *et al.*, 1992). The numbers of peripheral T cells and the ratio of CD4: CD8 cells were also comparable between $Fyn^{-/-}$ and WT mice in both studies. Collectively these data showed that Fyn, unlike Lck, was not essential to thymic development and peripheral repertoire generation.

When $Fyn^{-/-}$ thymocytes were activated with anti-CD3 antibodies plus PMA to provide signal 2, both groups reported reduced proliferation, intra-cellular calcium mobilisation and IL-2 production were reduced (Appleby *et al.*, 1992; Stein *et al.*, 1992). These defects could be overcome by stimulating with PMA and ionomycin that bypassed the requirement for TCR signalling. Work by Soriano

and colleagues also suggest that $Fyn^{-/-}$ mice showed defects in the deletion of thymocytes specific for the Mls-1^a self-super antigen (Stein *et al.*, 1992).

When the peripheral T cells were analysed both groups found that $Fyn^{-/-}$ splenic T cells recovered some degree of proliferative capacity compared to $Fyn^{-/-}$ SP thymocytes. However both studies showed that there was still a reduction in the response of splenic T cells from $Fyn^{-/-}$ mice compared to WT, albeit not as profound as that seen within the thymocyte populations. Intracellular calcium mobilisation and IL-2 production were still defective in peripheral $Fyn^{-/-}$ T cells. These data were obtained by activating the cells using antibodies against the CD3 complex with PMA. When other stimuli were used such as allogenic stimulator cells or SEA the proliferative defect of the $Fyn^{-/-}$ cells compared to WT was not as profound (Appleby *et al.*, 1992; Stein *et al.*, 1992). Together these data supported a role for Fyn as a positive regulator of T cell responses, with particular emphasis on calcium mobilisation and IL-2 production.

Work pre-dating the publication of the $Fyn^{-/-}$ mice using cell lines and transgenic over-expression strategies also supported the conclusion that Fyn played a positive role in T cell activation. For example over-expression of Fyn in T cell hybridomas resulted in attenuated IL-2 production (Davidson *et al.*, 1992). Later studies also in T cell lines suggested that Fyn may positively regulate IL-2 production (Fusaki *et al.*, 1994; Karnitz *et al.*, 1994). Furthermore transgenic mice expressing 20-fold more Fyn compared to control animals generated thymocytes that were hyper-responsive to anti-CD3 stimulation, whereas

expression of catalytically inactive Fyn lead to hypo-responsiveness in response to anti-CD3 stimulation (Cooke *et al.*, 1991).

In the initial $Fyn^{-/-}$ publications, both groups also conducted basic biochemical analysis of TCR induced phospho-tyrosine levels by stimulating cells with anti-CD3. Stein et al showed there was no change in the timing of phosphorylation or the identity of the proteins phosphorylated, however they did show a global reduction in the absolute phospho-tyrosine levels (Stein *et al.*, 1992). Appleby et al showed that Fyn deficiency led to the reduced phosphorylation of specific substrates while the tyrosine phosphorylation of other proteins remained comparable to those induced in cells from WT mice (Appleby *et al.*, 1992). These data provided biochemical evidence that Fyn was contributing to the induction of TCR mediated phosphorylation events.

Further biochemical analysis using Jurkat cell lines transfected with either Lck or Fyn suggested that Fyn was able to regulate a distinct pattern of protein phosphorylation with respect to Lck. The phospho-protein pattern observed in Fyn transfected cells resembled that induced by altered peptide ligands (Denny *et al.*, 2000). In concordance with this theory, $Fyn^{-/-}$ cells have been shown to be defective in their response to weak TCR agonists (Utting *et al.*, 1998). Furthermore, stimulation of T cells with TCR antagonists has been shown to preferentially lead to the activation of Fyn ((Huang *et al.*, 2000b; Huang *et al.*, 2000a). The conclusions drawn from these data was that Fyn was able to mediate positive signals downstream of the TCR via pathways that may be qualitatively and/or quantitativly different from Lck.

The role of Fyn in T cell responses to pMHC has not been extensively addressed. As discussed, the initial description of T cell activation events in the absence of Fyn involved activating polyclonal cells with anti-CD3 plus PMA. Anti-CD3 and PMA primarily activate through the TCR with no involvement of co-receptor molecules such as CD4 and CD8, which are known to associate with Lck (Barber *et al.*, 1989; Veillette *et al.*, 1988). Furthermore stimulation with MHC-peptide in the context of an APC will also involve a number of other co-stimulatory pathways. Fyn has been implicated in the signalling pathways downstream of a number of these co-stimulatory receptors, these include SLAM (Chan *et al.*, 2003), CD2 (Fukai *et al.*, 2000) and CD28 (Raab *et al.*, 1995). Physiological stimulation conditions would presumably involve ligation of not just the TCR, but also the panoply of receptors and ligands found on the T cell and APC, and may therefore utilise Fyn differently from antibody stimulation solely through the TCR.

The aim of this study was to determine the role of Fyn in peripheral T cell activation using pMHC and APCs, in contrast to anti-CD3 stimulation. To this end the $Fyn^{-/-}$ mice described by Stein et al were crossed onto the class I restricted F5 TCR transgenic line. T cells expressing the F5 TCR can be identified by using the Vβ11-specific antibody OKT-1. The F5 TCR recognises the NP peptide (amino acids 366 – 374) of the influenza A virus A/NT/60-68 (H3N2) in the context of $H2\text{-}D^{b}$ (Mamalaki *et al.*, 1993b; Mamalaki *et al.*, 1992). $Fyn^{-/-}$ F5 mice crossed onto a $Rag\text{-}1^{-/-}$ background have a monoclonal CD8 T cell repertoire that can be activated using the NP68 peptide. For clarity, I will refer to the $Rag\text{-}1^{-/-}$ F5

Fyn sufficient mice as F5 WT mice and the Rag-$1^{-/-}$ F5 $Fyn^{-/-}$ mice as F5 $Fyn^{-/-}$ mice.

3.2 Fyn deficiency does not alter CD8 T cell development in F5 mice

The development of F5 T cells and the response to cognate antigen has been well characterised (Smyth *et al.*, 2002; Mamalaki *et al.*, 1992). Introduction of the F5 TCR transgene into Rag-$1^{-/-}$ mice exclusively leads to positive selection of the CD8SP lineage (Mamalaki *et al.*, 1993b). As expected from the previous $Fyn^{-/-}$ reports selection into the CD8SP lineage proceeded normally in the absence of Fyn with no CD4SP evident (Figure 3.1), furthermore the frequency of DP cells was also comparable to WT F5 mice. Within the CD8SP population there are immature CD8ISP and mature CD8SP, which can be distinguished by TCR expression levels whereby cells that have up-regulated their TCR levels are mature CD8SP cells (Schrum *et al.*, 2003). Figure 3.1A shows that maturation of CD8SP cells, as judged by TCR levels expressed on CD8SP, proceeded normally in the absence of Fyn. Figure 1B shows that the absolute numbers of DN, DP and mature CD8SP was also comparable between WT F5 and $Fyn^{-/-}$ F5 thymi. More detailed analysis of the DN population on the basis of CD25 and CD44 expression revealed that there was a small but significant increase in the frequency (Figure 3.1C) and absolute number (Figure 3.1D) of DN3 cells in the thymi from $Fyn^{-/-}$ F5 mice, whereas other DN populations were comparable. Therefore, while the maturation of CD8SP was normal, Fyn did seem to play a role at the DN3 to DN4 transition, even in the presence of Lck.

Analysis of the peripheral lymphoid organs of these mice revealed that in both groups the frequency of $CD8^+$ $V\beta11^+$ in the PLN was ~90% (Figure 3.1E). However, the absolute numbers of $CD8^+V\beta11^+$ cells were elevated in the PLN of F5 $Fyn^{-/-}$ mice compared to *F5 WT* mice (Figure 1F). Expression of CD62L, which is cleaved and shed by activated T cells (Chen *et al.*, 1995; Tedder *et al.*, 1995) , was comparable between both groups and CD5 levels were also identical (Figure 1E), as were the expression levels of CD25 and CD69 (data not shown).. Despite the increase in the number of peripheral T cells from F5 $Fyn^{-/-}$ looked phenotypically normal compared to F5 WT cells and typically naïve, as judged by the expression of activation markers.

3.3 Fyn deficiency increases cell division and CD25 expression but not triggering or cell cycle entry

Upon stimulation with cognate antigen, a naïve CD8 T cell will become activated and upregulate a number of cell markers, before entering into a programme of cell division and differentiation. In order to address the role of Fyn in the initiation of an antigen specific CD8 T cell response *in vitro*, proliferation and expression of activation markers was examined by flow cytometry. Upon T cell activation, one of the earliest markers to be expressed is the type II membrane glycoprotein CD69 (Castellanos *et al.*, 1997; Hamann *et al.*, 1993). Although there has been no function assigned to this molecule, it is considered to reflect the activation status of a cell as a result of TCR triggering (Zola, 2000). At 24 hrs after primary stimulation, T cells have not yet entered division, therefore CD69 expression was assessed as a measure of early cell activation in the absence of Fyn. Figure 3.2A

shows bi-variant FACS plots for three concentrations of NP68 for each group in order to highlight how gating and analysis was performed. These FACS data were then used to generate Figure 3.2B by plotting the percentage of CD69+ cells versus NP68 concentration. From the data in Figure 3.2B it is clear that F5 $Fyn^{-/-}$ T cells behave the same as F5 WT cells over a range of peptide concentrations, as judged by the induction of CD69 expression.

Activation of CD8 T cells also induces the expression of the surface receptor CD25. CD25 is the α subunit of the IL-2 receptor, and in concert with the β (CD122) and γ (CD132) subunits forms the high affinity complex (Leonard *et al.*, 1990). CD25 expression has a functional consequence for the T cell enabling it to respond to IL-2 (Leonard and Lin, 2000). Figure 3.2C shows that the MFI of CD25 expression was comparable in both groups of cells at 24 hrs. Also the percentage of Fyn deficient F5 CD8 T cells that had upregulated CD25 at 24 hrs in response to a titration of NP68 was identical to controls (Figure 3.2D).

In order to assess the proliferative capacity, cells were labelled with CFSE and activated for various times with a titration of NP68 peptide. Two informative parameters were determined from the analysis of the CFSE division profiles. First we determined the proportion of cells that were triggered to divide at a particular antigen concentration (% divided). Second we calculated the average number of divisions a cell underwent once it had been triggered (burst size). In both cases the parameters are adjusted to relate back to the starting cell population by correcting for cellular divisions (Gett and Hodgkin, 2000). By 48 hrs both F5 WT and $Fyn^{-/-}$ cells had divided as shown by the dilution of CFSE (Figure 3.3A).

Analysing the data in Figure 3.3A revealed that the proportion of cells entering division (Figure 3.3B) and the number of divisions they carried out (Figure 3.3C) were comparable. In contrast to the CFSE data, analysis of CD25 expression at 48 hrs revealed that in the absence of Fyn, the MFI of this marker was elevated ~ 2-fold compared to F5 WT cells (Figure 3.3D). Furthermore, the percentage of F5 $Fyn^{-/-}$ cells expressing CD25 was increased by ~3-fold at a dose of 10^{-2} μM NP68 (Figure 3.2D and E).

When division was assessed at 72 hrs, as shown in Figure 3.4B, it was clear that F5 $Fyn^{-/-}$ cells had undergone an extra round of division compared to F5 WT over a range of peptide concentrations. The increase in the burst size is illustrated in the CFSE histograms, as they show a greater frequency of cells in the later division peaks in the absence of Fyn (Figure 3.4A). Analysis of CD25 expression at 72 hrs showed that the increased expression in the absence of Fyn was sustained both in terms of the MFI (Figure 3.4C) and the percentage of cells expressing CD25 (Figure 3.4D) compared to F5 WT cells.

3.4 $Fyn^{-/-}$ and WT F5 cells have similar activation kinetics

One possibility to explain the elevated expression of CD25 observed at 48 hrs in the absence of Fyn could be due to altered kinetics of the response. For example, if CD25 expression peaked earlier in F5 WT cells than the F5 $Fyn^{-/-}$, at later time points the expression by F5 WT cells would already be in decline and the F5 $Fyn^{-/-}$ response would appear elevated. Therefore the expression levels of CD25 and CD69 before 24 hrs was analysed to address this issue. To this end Fyn deficient

and WT F5 T cells were activated using a single dose of NP68 peptide and analysed for CD69 expression (Figure 3.5A) and CD25 expression (Figure 3.5B) at the indicated time points. In both groups CD69 expression was comparable and was first detected at 1 hr, peaked between 3 and 24 hrs and then declined at 48 hrs. As with CD69, prior to 24 hrs, CD25 expression was equivalent in the absence of Fyn, however from the data in Figure 3.5B it is clear that CD25 expression remained elevated at 48 hrs. In contrast to this, CD25 expression in the Fyn sufficient group dropped by 50% at 48 hrs. By 72 hrs CD25 expression in both groups was comparable. Therefore, the observed elevation of CD25 expression in the F5 $Fyn^{-/-}$ T cells was not due to delayed kinetics of the response.

3.5 $Fyn^{-/-}$ F5 T cells produce more IL-2 in response to NP68 stimulation

In order to determine why F5 $Fyn^{-/-}$ CD8 T cells show prolonged CD25 expression and an increased burst size, the production of IL-2 by these cells was measured. TCR derived signals drive the initial expression of CD25 and IL-2 by the T cell (Kim and Leonard, 2002). However, the level of CD25 is then maintained on the surface of the cell by a classical feedback mechanism whereby IL-2 binds the high affinity receptor activating STAT5 leading to further CD25 expression (Kim *et al.*, 2001; Smith and Cantrell, 1985). It seemed plausible that prolonged expression of CD25 observed in the absence of Fyn was a direct result of the capacity of these cells to make IL-2. Therefore IL-2 production by Fyn deficient F5 CD8 T cells compared to F5 WT cells was determined using a number of different techniques. Figure 3.6A shows IL-2 production by antigen activated $CD69^{+}$ F5 CD8 cells as measured by a FACS based assay detecting secreted IL-2

protein at the single cell level. When these data were plotted in graphical form, it was clear that the initiation of IL-2 production in both groups was comparable (Figure 3.6B). However the percentage of $CD69^+$ F5 $Fyn^{-/-}$ CD8 T cells able to make IL-2 was elevated by ~ 2-fold at 6 hrs and at every time point measured thereafter compared to F5 WT cells. The increased production of IL-2 was also confirmed by measuring the amount of cytokine in the supernatants after 48 hrs of culture using the CTLL-2 bioassay, this is shown in the insert panel of Figure 3.6B. There was a 10-fold increase in IL-2 production in supernatants from F5 $Fyn^{-/-}$ cell cultures compared to F5 WT samples. Therefore, increased production of IL-2 by F5 $Fyn^{-/-}$ correlated with increased accumulation in the media. To confirm these observations, real time PCR for steady-state IL-2 mRNA was performed. IL-2 mRNA levels were elevated ~2 fold in the Fyn deficient F5 T cells activated with NP68 (Figure 6C). These data show that mRNA production peaked in both groups at 4 hrs, which was one hr before peak production measured using the IL-2 capture assay (Figure 3.6B), therefore mRNA and protein production correlated well. Furthermore, from the profile of IL-2 mRNA transcription it was unlikely that an increase in IL-2 message stability was responsible for the elevated IL-2 in the absence of Fyn, as levels began to decrease with similar kinetics in both groups (Figure 3.6C). The capture assay and real time PCR data suggested that IL-2 production by activated CD8 T cells occurred between 3 – 10 hrs after activation, which is prior to entry into cell division. Upon recall stimulation with PdbU, ionomycin and with Brefeldin A present to block protein export, Fyn deficient cells again produced more IL-2 than F5 WT cells (Figures 3.6D and 3.6E). Intracellular staining was maximal at 24 hrs in both groups with a gradual decline over 48 and 72 hrs. At 48 and 72 hrs the

MFI values for IL-2 staining in the absence of Fyn were ~2.5 times those of control values (Figure 3.6E). Additionally, F5 Fyn$^{-/-}$ cells retained the ability to produce more IL-2 than F5 WT cells throughout each division peak (Figure 3.6D). In all experiments, prior activation with NP68 peptide was required in conjunction with restimulation to detect IL-2 by intracellular staining as PdbU, ionomycin and Brefeldin A alone for 4 hrs gave no signal (data not shown).

3.6 Prolonged CD25 expression by F5 Fyn$^{-/-}$ cells is a direct result of increased IL-2 production

It was possible that the increase in IL-2 production seen in the absence of Fyn was not the direct or only cause of the prolonged CD25 expression, but rather that there were defects in the processes to down modulate this receptor. The next set of experiments was designed to answer this question. The strategy employed was two fold. Firstly, the availability of IL-2 in the culture well was blocked using the anti-IL-2 antibody S4B6 to determine whether the expression of CD25 on the F5 Fyn deficient cells could be reduced to that of F5 WT cells. Secondly, cultures were spiked with an excess of IL-2 to determine if CD25 expression on F5 WT cells could be increased to the levels seen in the absence of Fyn. The expression of CD69 is not influenced by IL-2 (Nakajima *et al.*, 1997) unlike CD25, and served as a control in these experiments. From the graphs in Figures 3.7B and 3.7D it was clear that blocking IL-2 or spiking with IL-2 respectively had no influence on the expression of CD69 in either group. In contrast IL-2 manipulation had a profound influence on CD25 expression. Blocking IL-2 (Figure 3.7A) reduced the expression of CD25 in the F5 Fyn$^{-/-}$ group to a level

similar to that of untreated F5 WT cells at 48 hrs, and completely at 72 hrs. At 24 hrs blocking IL-2 had no effect on CD25 expression, this was expected as initial CD25 expression is driven by TCR signals and is independent of IL-2 (Nakajima *et al.*, 1997). Therefore prolonged CD25 expression seen in the absence of Fyn was a direct result of IL-2 availability in the culture wells. Interestingly, blocking IL-2 in the culture was unable to reduce expression of CD25 on the surface of F5 WT cells further. This implies that the small quantity these cells were making was probably immediately utilised and not secreted into the media. This conclusion is supported by the CTLL-2 assay data (Figure 3.6B, insert panel) showing that IL-2 content in the media of control cells at 48 hrs was very low. The converse experiment of spiking cultures with IL-2 revealed that CD25 expression by the F5 $Fyn^{-/-}$ cells could not be elevated further (Figure 3.7B). This suggested that production and response to this cytokine was already maximal. Addition of IL-2 to F5 WT cells was able to prolong CD25 expression but was unable to achieve levels seen in the Fyn deficient group. This result may reflect a functional advantage that autocrine IL-2 consumption may have over paracrine or it may suggest that F5 $Fyn^{-/-}$ cells can respond better to the presence of IL-2 than F5 WT cells.

3.7 Increased IL-2 levels improve cell survival of F5 $Fyn^{-/-}$ cells increasing the burst size at 72 hrs.

IL-2 has pleotropic effects on a T cell and it is thought to play a role in sustaining cell division either by increasing cell cycling or by improving cell survival (Van Parijs *et al.*, 1999). We wanted to address if the increased IL-2 production by F5

Fyn$^{-/-}$ was also responsible for the increased burst size at 72 hrs. The mechanism by which faster cell cycling could affect the bursts size values is clear, in that increased cycling would lead to more cells undergoing more rounds of division. However cell survival could also affect the burst size value independently of a cells ability to cycle. If more cells within a division peak survive long enough to undergo the next round of division, this would lead to an exponential increase in cell numbers significantly increasing the burst size. Conversely, if a cell population showed decreased survival potential then this would lower the probability of cells undergoing further rounds of division, decreasing burst size values. To answer these questions the frequency of apoptotic cells within each round of cell division was measured at 72 hrs in cell populations with or without Fyn that had been CFSE labelled and activated with NP68. Additionally, to further elucidate the role of IL-2 in this process the IL-2 blocking antibody S4B6 was included in some samples. Firstly, from the data in Figure 3.8A, it is clear that without any IL-2 manipulation (hatched bars) there were more live cells in the division peaks of CFSE labelled NP68 activated F5 Fyn$^{-/-}$ cells (red hash) compared to the controls (blue hash). The survival advantage was particularly evident in the second and third rounds of division. The inclusion of S4B6 was able to eliminate the survival advantage of Fyn deficient cells, reducing the frequency of Annexin V negative cells to a comparable level with F5 WT cells without S4B6. The correlation of these data with the burst size values is striking. Blocking IL-2 in the cultures containing F5 Fyn$^{-/-}$ cells also reduced the burst size values to exactly that of F5 WT cells without any IL-2 manipulation (Figure 3.8B).

These data show that IL-2 was responsible for the elevated burst size at 72 hrs, however from the data in Figure 3.5B it is clear that maximal IL-2 levels would be present in the supernatants at earlier time points. It is therefore unclear why burst size values at 48 hrs were comparable between F5 WT and $Fyn^{-/-}$ (Figure 3.3C) if more IL-2 is clearly available to the F5 $Fyn^{-/-}$ cells. Classically IL-2 is thought of as a T cell mitogen (Morgan *et al.*, 1976) and has been shown to drive cell cycle entry and progression via STAT5 signalling (Moriggl *et al.*, 1999). It was interesting, therefore, that F5 $Fyn^{-/-}$ cells did not show increased propensity to enter division even though IL-2 levels were elevated at 48 hrs. To confirm that IL-2 could not influence the burst size values at 48 hrs the same strategy of blocking IL-2 with the S4B6 antibody was employed. From Figure 8C it is clear that blocking IL-2 has no effect on the burst size in either group. The addition of a large excess of recombinant IL-2 also had no effect (data not shown). Furthermore the addition of a large excess IL-2 could not influence the percent divided parameter at 48 hrs in either group (Figure 3.8D). The reciprocal experiment using blocking antibody also had no effect on cell entry into division (data not shown). Collectively, these data suggest that initial entry into and the initial progression of the CD8 division programme is independent of IL-2, but becomes dependent thereafter.

3.8 The activation phenotype of $Fyn^{-/-}$ cells depends upon the method of stimulation

It has been previously reported that Fyn deficient T cells were hypo-responsive to stimuli and failed to produce IL-2 (Appleby *et al.*, 1992; Stein *et al.*, 1992). In

contrast the data presented here shows that F5 Fyn deficient cells were instead hyper-responsive producing elevated amounts of IL-2 compared to F5 WT cells. There are a number of possible explanations for this result. Firstly the original studies investigated the response of a polyclonal repertoire of splenic CD4 and CD8 cells, whereas the F5 system has monoclonal population of CD8 T cells. Secondly, the F5 system allowed activation with pMHC and APCs whereas polyclonal cells were activated using anti-CD3 antibodies plus PMA with no co-receptor involvement. Therefore, the next set of experiments was designed to establish whether these discrepancies arose from the different stimuli employed or from the use of a monoclonal versus polyclonal T cell repertoire. In the original publications, stimulations were carried out using the mAb 2C11. The clone 2C11 is specific for the epsilon chain of the CD3 complex and has been shown to induce T cell activation in a dose dependent manner (Leo *et al.*, 1987). In order to provide signal 2 to the T cell, PMA was also included in the stimulation conditions (Weiss *et al.*, 1986). We wanted to determine if stimulation in this manner with 2C11 would lead to hypo-responsiveness or hyper-resposiveness of F5 $Fyn^{-/-}$ cells.

To address this question, stimulations were carried out using antibodies immobilised on micro-beads to act as artificial APC. The advantage of this system over soluble antibodies and APC is that stimulating antibodies become fixed on a surface thus preventing internalisation and signal termination, and it is possible to control which receptors are engaged. In these sets of experiments anti-CD28 antibody was used to provide signal 2, however PMA also gave the same result (data not shown). The data in Figure 3.9A shows that stimulation of Fyn

deficient F5 CD8 T cells with 2C11 and anti-CD28 antibodies (broken lines) immobilised on the surface of a micro-bead led to a reduction in the % divided at 48 and 72 hrs compared to F5 WT cells. Figure 3.9B shows that the burst size of F5 Fyn$^{-/-}$ cells was also reduced compared to F5 WT cells, as was the percentage of CD25 expressing cells (Figure 3.9C) and IL-2 expressing cells (Figure 3.9D). Therefore F5 Fyn$^{-/-}$ cells showed a similar hypo-responsive phenotype reported for the polyclonal cells when stimulated through the TCR and CD28.

One significant difference between an anti-CD3 stimulation versus pMHC stimulation is the involvement CD8 co-receptor in the latter. Moreover a single pMHC molecule will not only bind to the TCR on the surface of the responding T cell, it will also engage the CD8 co-receptor (Gao *et al.*, 1997; Salter *et al.*, 1990). Lck has been shown to be associated with the cytoplasmic tail of CD8 (Veillette *et al.*, 1988) and engagement of MHC-peptide will bring Lck into the proximity of the TCR /CD3 complex. In order to determine whether the hyper-responsiveness of F5 Fyn$^{-/-}$ cells to pMHC stimulations was influenced by co-engagement of the TCR and CD8, micro-beads with immobilised anti-CD3, CD28 and CD8 antibodies were used. Figure 3.9A shows that inclusion of the anti-CD8 antibody (solid lines) in the stimulus restored the ability of F5 Fyn$^{-/-}$ to enter division compared to F5 WT cells. Furthermore, inclusion of anti-CD8 antibody also elevated the burst size of F5 Fyn$^{-/-}$ cells (Figure 9B), it was also able to increase CD25 expression (Figure 3.9C) and, most significantly, induce elevated IL-2 production (Figure 3.9D) compared to F5 WT cells activated in the same manner. Therefore, inclusion of anti-CD8 antibody into the stimulus, along with anti-CD3

was able to fully mimic the hyper-responsive phenotype seen with antigen in the absence of Fyn.

3.9 Increased production of IL-2 by F5 Fyn$^{-/-}$ cells is regulated by TCR and CD8 engagement alone

As well as CD8 co-ligation with the TCR, soluble NP68 peptide activation of whole F5 lymph node preparations will involve the subsequent engagement of multiple co-receptors on the T cell and APC. This is an important consideration as Fyn has been implicated in the signalling processes of a number of different receptors on the T cell such as CD2 (Fukai *et al.*, 2000), CD45 (Shiroo *et al.*, 1992) and SLAM (Chan *et al.*, 2003) the ligands of which are expressed on the APC. Although activation through the TCR and CD8 was able to induce F5 Fyn$^{-/-}$ to produce elevated IL-2 levels it could not be excluded that other receptors contributed during antigen recognition. To confirm that the only requisite for inducing elevated IL-2 levels in the absence of Fyn during antigenic stimulation was co-ligation of CD8 and TCR complexes by pMHC complexes, stimulations were carried out using highly purified F5 CD8 T cells from both groups (~99.6% TCR+ CD8+ cells). These cells were placed onto an immobilised layer of NP68 loaded MHC-dimer molecules for defined time points. The pMHC dimer molecules will only signal through the TCR and CD8 molecules on the T cell surface and have been shown to drive activation without a need for signal 2 (Goldstein *et al.*, 1998). Stimulation of purified F5 Fyn$^{-/-}$ cells on the MHC-peptide layer led to identical % divided values but, as after APC-Peptide stimulations, elevated IL-2 production (Figure 3.10A and 3.10B). Collectively

these data showed that activation using MHC-dimer molecules in the absence of co-stimulatory APC interactions were able to mimic the response of F5 Fyn$^{-/-}$ cells activated by peptide in the presence of APC.

The contribution of APC derived co-stimulation to the hyper-responsive phenotype of the F5 Fyn$^{-/-}$ cells was further addressed using soluble 2C11 as a stimulus. Activation in this manner requires APCs to bind the Fc portion of the antibody via Fc receptors (Leo *et al.*, 1987). This will lead to stimulation via the TCR and other co-stimulatory molecules, however CD8 will not be brought into close proximity with the TCR as the self-MHC class I molecules will not be stimulatory without loaded peptide. Stimulations using 2C11-loaded APCs lead to a reduction in CD69 expression and IL-2 production respectively by F5 Fyn$^{-/-}$ T cells (Figures 3.10C and 3.10D). This showed that stimulation via the TCR and other co-stimulatory molecules expressed on the APC were not able to induce hyper-responsiveness of F5 Fyn$^{-/-}$ cells, and instead resembled stimulation through the TCR alone.

CD28 has been shown to play a role in increasing IL-2 production by activated T cells (Fraser *et al.*, 1991; Jenkins *et al.*, 1991). CD28 is expressed on T cells and binds to CD80 (B7-1) and CD86 (B7-2) located on the surface of the APC (Collins *et al.*, 2002; Green *et al.*, 1994). Ligation of CD28 leads to increase IL-2 mRNA by elevating the transcriptional activity of the *Il2* promoter through the CD28RE (Diehn *et al.*, 2002; Shapiro *et al.*, 1997) and also by regulating signals that improve stability of the transcripts (Umlauf *et al.*, 1995). Stimulations using micro-beads also included the use of an anti-CD28 antibody, furthermore

activated CD8 T cells have been shown to express the CD28 ligands B7-1 and B7-2 (Hathcock *et al.*, 1994). Therefore, even after APC depletion it could not be fully excluded that the CD28 pathway was not playing a role in the increased IL-2 levels produced by F5 $Fyn^{-/-}$ cells. It should also be noted that Lck and Fyn have been implicated in CD28-mediated signalling (Holdorf *et al.*, 1999; Raab *et al.*, 1995). To address the role of CD28 pathways in inducing elevated IL-2 production in the absence of Fyn we exploited the fact that B7-1/2 - CD28 interaction is antagonised by the molecule CTLA-4. This molecule can bind to B7-1/2 with a higher affinity than CD28 (Tivol *et al.*, 1995). To this end, cells were pre-incubated with 10 μg /ml of a CTLA-4 Ig molecule previously shown to inhibit CD28 signalling at the level of CD28RE activity of the Il-2 promoter (Shapiro *et al.*, 1998). If the increased IL-2 in the absence of Fyn was due to CD28 signals then blocking with CTLA-4Ig should reduce production to WT levels. The data in Figure 6.11A shows that inhibition of CD28 co-stimulation was able to reduce IL-2 production in both groups by ~28%, however F5 $Fyn^{-/-}$ cells were still able to produce more IL-2 than F5 WT cells (data not shown).

To further confirm that co-stimulation through CD28 was not responsible for the F5 $Fyn^{-/-}$ phenotype, the ability of peptide pulsed APC or pMHC dimers and no APC to induce IL-2 mRNA was compared. Figure 3.11B shows that *Il2* gene transcription was up-regulated in F5 $Fyn^{-/-}$ cells compared to F5 WT cells, regardless of the stimulation method employed. Stimulation with APC served to upregulate IL-2 mRNA in both groups. Therefore, it was concluded that CD28 signals were able to increase IL-2 production in both stes of cells, but did not

contribute to the ability of F5 Fyn$^{-/-}$ cells to make elevated IL-2 levels compared to F5 WT cells.

3.10 Polyclonal Fyn$^{-/-}$ CD8+ cells produce more IL-2 after TCR and CD8 crosslinking

If F5 Fyn$^{-/-}$ cells were activated using MHC-peptide they were hyper-responsive whereas published data using polyclonal Fyn$^{-/-}$ cells showed that these cell were hypo-responsive to an anti-CD3 antibody stimulus. The data in figures 3.9 and 3.10 indicate that these discrepancies were due to the method of stimulation and not due to differences in the cell type and specificity. Therefore, if this conclusion was correct, stimulating polyclonal Fyn$^{-/-}$ cells using a method that cross-linked the TCR and CD8 should induce hyper-responsiveness and mimic the pMHC response. Naïve CD8 cells (CD62L high) were obtained from polyclonal Fyn$^{-/-}$ mice and WT controls (C57BL/6 mice) using a cell sorter. In all experiments purity in both groups was greater than 97% (TCR^{+}, CD8^{+} CD62L^{hi}, CD44^{lo}). Stimulations were carried out using beads coated with different combinations of immobilised stimulatory antibodies as described for Figures 3.10A and 3.10B.

When naïve polyclonal CD8 cells were activated using beads coated with anti-CD3 and anti-CD28 antibodies only, analysis of cell division and IL-2 production at 72 hrs revealed that Fyn deficient cells were hypo-responsive to this stimulus compared to WT cells in terms of the burst size (Figure 3.12A, left panels). This was in concordance with the published data on polyclonal Fyn$^{-/-}$ cells, showing reduced proliferation. However, stimulation with beads that included an anti-CD8

antibody in conjunction with anti-CD3 and anti-CD28 antibodies increased the ability of $Fyn^{-/-}$ CD8 cells to divide compared to WT cells, specifically elevating the burst size values (Figure 3.12A, right panels). Stimulation using beads coated with anti-CD3, CD28 and CD8 antibodies also led to an increase in the production of IL-2 by the $Fyn^{-/-}$ CD8 cells (Figure 3.12B). Therefore, when $Fyn^{-/-}$ polyclonal CD8 were stimulated via the TCR and CD8 the response mimicked the hyper-responsiveness induced by antigen.

3.11 Discussion

The aim of the work presented in this chapter was to establish the role of Fyn in the activation of peripheral CD8 T cells by MHC-peptide and APC. To this end, $Fyn^{-/-}$ mice were back-crossed with mice expressing the class-I restricted F5 TCR transgene on a $Rag\text{-}1^{-/-}$ background, generating a monoclonal CD8 T cell repertoire that would respond to cognate antigen.

Thymic development in the F5 $Fyn^{-/-}$ mice proceeded normally and generated a peripheral repertoire of CD8 T cells comparable to F5 WT controls. When LN cells were activated using the NP68 peptide the threshold of activation was the same in both groups, however F5 $Fyn^{-/-}$ cells produced elevated levels of IL-2 at early time points. The increase in IL-2 production led to elevated expression of CD25 on the cell surface and improved cell survival, which in turn increased the proliferative capacity of the F5 $Fyn^{-/-}$ compared to F5 WT cells at later time points. The hyper-responsiveness of the monoclonal $Fyn^{-/-}$ cells was different from the activation phenotype of polyclonal $Fyn^{-/-}$ cells reported by Stein et al and

Appleby et al. In those studies, $Fyn^{-/-}$ cells were hypo-responsive to stimuli that primarily engaged the TCR alone. When we stimulated the F5 $Fyn^{-/-}$ cells with anti-CD3 alone we obtained similar results. However, the use of stimuli that were able to engage both the TCR and CD8 molecules induced the hyper-responsive phenotype in both F5 $Fyn^{-/-}$ and the polyclonal CD8+ $Fyn^{-/-}$ cells compared to WT controls. Moreover, we could exclude that this hyper-responsive phenotype resulted from stimulation of any receptors other than the TCR and CD8 either by removing APC and engaging the TCR and CD8 directly with antibodies or MHC-dimers, or by directly antagonising engagement of other receptors. Collectively these data suggest that Fyn can act as a negative regulator of T cell activation, by regulating IL-2 production by antigen activated CD8 T cells.

The reason for the different outcomes induced after anti- CD3 versus anti-CD3/8 or antigen stimulation of $Fyn^{-/-}$ cells may be explained by the nature in which the different stimuli engage Src kinases. Our data, in concordance with Stein et al and Appleby et al, indicate that Fyn plays a positive role in transducing signals via the TCR. Moreover three recent reports have also shown that stimulation of $Fyn^{-/-}$ cells with ant-CD3 led to general defects in T cell activation (Cannons *et al.*, 2004; Davidson *et al.*, 2004; Sugie *et al.*, 2004). Furthermore, it has been proposed that Fyn is important for T cell responses to stimuli such as weak TCR agonists (Utting *et al.*, 1998) and antagonists (Huang *et al.*, 2000b; Huang *et al.*, 2000a). These altered peptide ligands do not activate T cells as efficiently as a full agonist ligands, potentially due to reduced activation of the Lck substrate ZAP-70 compared to levels induced by full agonistic ligands (Reis e Sousa *et al.*, 1996; Madrenas *et al.*, 1995; Sloan-Lancaster *et al.*, 1994). Within a T cell the

CD8-associated Lck kinase activity has been shown to be important to T cell responses (Irie *et al.*, 1998; Irie *et al.*, 1995) and therefore anti-CD3 cross-linking in the absence of CD8 may not utilise this Lck activity efficiently, resulting in signals similar to those induced by the low affinity peptides. With CD3 stimulation, weak TCR ligands may preferentially signal through the TCR and Fyn. Furthermore, the observations that Fyn can associate with components of the CD3 complex (Timson Gauen *et al.*, 1992; Samelson *et al.*, 1990), and that TCR stimulation in the absencse of Fyn leads to a reduction in phosphorylation of downstream proteins (Fusaki *et al.*, 1994) provides some biochemical evidence that the TCR may have a dependency on Fyn for signal transduction.

In contrast to anti-CD3 alone, activation with MHC-peptide molecules or cross-linking antibodies that were able to bring the TCR and CD8 into close proximity with one another overcame the triggering defect in both the monoclonal and polyclonal $Fyn^{-/-}$ cells. Several other groups showed little or no defect in $Fyn^{-/-}$ cells in response to stimuli able to engage both CD8 and TCR molecules into close proximity (Utting *et al.*, 1998; Liao *et al.*, 1997). Furthermore, both Stein et al and Appleby et al showed that stimulation with allogeneic stimulator cells (MLR) was capable of inducing improved activation of $Fyn^{-/-}$ cells compared to anti-CD3 alone. Interestingly, MLR induced proliferation by $Fyn^{-/-}$ cells may even have surpassed that of WT control cells in one study (Stein *et al.*, 1992) indicating that $Fyn^{-/-}$ polyclonal cells may also be hyper-responsive to antigen.

The cytoplasmic domain of CD8 has been shown to associate with LAT (Bosselut *et al.*, 1999), LIME (Brdickova *et al.*, 2003; Hur *et al.*, 2003) and Lck (Veillette *et*

al., 1988) all of which could in theory circumvent the need for Fyn-mediated activation signals. We favour the idea that CD8 associated Lck is responsible for overcoming the hypo-responsive T cell activation in $Fyn^{-/-}$ cells after TCR stimulation alone. It has long been established that co-ligating CD8 with the TCR enhances T cell activation (Jonsson *et al.*, 1989; Boyce *et al.*, 1988). Moreover, Lck can phosphorylate the ITAM residues in the CD3 complex (van Oers *et al.*, 1996a; Wange *et al.*, 1995; Iwashima *et al.*, 1994) and activate ZAP-70 (Kong *et al.*, 1996; Chan *et al.*, 1995) thus mediating a number of the proximal signalling events in a T cell that after TCR stimulation alone may be defective in the absence of Fyn.

These data raise the question of how bringing CD8 to the TCR could lead to elevated IL-2 levels in the absence of Fyn. Certainly, several previous reports state that stimulation of $Fyn^{-/-}$ primary cells or cell lines expressing mutant Fyn molecules led to reduced IL-2 production (Fusaki *et al.*, 1994; Appleby *et al.*, 1992; Stein *et al.*, 1992). Reciprocally, over expression of Fyn then greatly attenuated IL-2 production (Fusaki *et al.*, 1994; Davidson *et al.*, 1992). However, these studies were either conducted in cell lines, which may be co-receptor independent or if primary cells were used, then the method of stimulation may not have effectively activated Lck. Furthermore, the activity of Lck and Fyn are regulated by the phosphorylation status of the positively regulating tyrosine residues within the kinase domains (Tyr394 in Lck, and Tyr417 in Fyn) as well as the inhibitory tyrosine residues located in the C-termi (Tyr505 Lck and Tyr529 in Fyn) (Yamaguchi and Hendrickson, 1996). Dephosphorylation of the Tyr residues that promote kinase activity is mediated by phoshpatases such as PEP

(Cloutier and Veillette, 1999; Gjorloff-Wingren *et al.*, 1999), PtP-PEST (Mustelin and Tasken, 2003) and possibly CD45 (Burns *et al.*, 1994; Sieh *et al.*, 1993). Furthermore, phosphorylation of the inhibitory C-terminus Tyr residues is mediated by the PEP associated kinase Csk (Chow *et al.*, 1993; Bergman *et al.*, 1992; Okada *et al.*, 1991). These two molecules are brought into cellular proximity with Lck and Fyn through the actions of the transmembrane adapter molecule PAG. When PAG is phosphoryalted on Tyr317, Csk and associated PEP are able to form a protein complex that acts to down-regulate Lck and Fyn activity (see Figure 1.3). Fyn has been shown to associate constitutively with PAG via its SH3 domain (Brdicka *et al.*, 2000; Marie-Cardine *et al.*, 1999) and may be responsible for phosphorylating Tyr317 as in $Fyn^{-/-}$ thymocytes phosphorylation of this residue is defective (Yasuda *et al.*, 2002). Therefore, in the absence of Fyn, the inability to efficiently form the inhibitory PAG/CSK/PEP complex could disregulate Lck kinase activity. This could, in theory, lead to increased duration of Lck signalling and increase IL-2 production. Certainly, disregulated Lck mutants that lack the negative regulatory Tyr 505 when expressed in cell lines show attenuated IL-2 production after stimulation (Luo and Sefton, 1992).

Another molecule that has been shown to influence IL-2 production in T cells is the co-stimulatory molecule CD28 (Fraser *et al.*, 1991; Jenkins *et al.*, 1991). To address whether CD28 co-stimulation contributed to the elevated IL-2 production in the absence of Fyn, we antagonised the B7-1/2 interaction with anti-CTLA-4 Ig. The results suggested that CD28 signalling was responsible for ~30% of total IL-2 production in both groups, but not responsible for the increase in the absence

of Fyn. The effect of CD28 stimulation may have been mediated by changes in the transcriptional activity of the *Il2* gene, or by differences in stability of the IL-2 message as CD28 signals potentially control both (Sanchez-Lockhart *et al.*, 2004). Furthermore, previous studies have shown that CD28 stimulation combined with TCR activation led to a 30% increase in IL-2 production (Umlauf *et al.*, 1995), a figure consistent with our findings. Also, gene chip analysis of global expression patterns suggested that CD28 co-ligation with the TCR serves to induce quantitative changes rather than qualitative in gene transcription (Diehn *et al.*, 2002). However, both Lck and Fyn have been shown to be important to the phosphorylation of Tyr 191 withinin the cytoplasmic tail of CD28, mediating the binding of factors such as PI 3-kinase, Grb2 and Itk (Raab *et al.*, 1995). Therefore, the loss of Fyn, and the potential disregulation of Lck could in theory have altered CD28 signalling, however using the critera of IL-2 production to judge CD28 co-stimulation we observed no effect in the absence of Fyn in our system.

After *in vitro* activation, proliferation of CD8 T cells has been shown to peak after ~3 days and then decline. This was thought to be due in part to the elimination of antigen-pusled APC as CD8 cells became CTL after 3 days (Sprent and Schaefer, 1989). However, stimulation of CD8 cells using artificial stimuli that could not be eliminated by CTL activity were also shown to lead to a loss of proliferative capacity after 3 days (Deeths and Mescher, 1997). Studies have shown that the decline in CD8 proliferation corresponds with the loss of the ability of these cells to produce IL-2, even after re-stimulation. This phenomenon has been termed Activation Induced Non-Responsiveness (AINR) (Deeths *et al.*, 1999). Our data

supports this concept, as after primary stimulation, IL-2 production measured by the capture assay became undetectable by ~10 hrs. Although IL-2 production could still be detected at 24 hrs after re-stimulation with PdbU and ionomycin, by 72 hrs even such recall stimulations became ineffective in both groups suggesting that they had become refactory to restimulation and possibly undergone AINR. Furthermore, as PdbU and ionomycin bypass the need for TCR signalling, this supported the idea that whatever the mechanism responsible for down-regultaing IL-2 production after restimulation, it had been established by the initial pMHC driven signals.

In the absence of Fyn the initiation of IL-2 protein secretion measured by capture assay occurred with similar kinetics to F5 WT controls, however the magnitude and duration of production was increased. This correlated with improved survival and proliferation of F5 $Fyn^{-/-}$ cells after 3 days in culture. As IL-2 is important for CD8 cell survival, it has been proposed that the induction of AINR acts as a mechanism to limit expansion because CD8 cells will die by passive mechanisms when IL-2 is unavailable (Tham *et al.*, 2002). Therefore AINR will regulate the proliferative response of CD8 cells by setting the production and availability of IL-2. It is plausible therefore that Fyn influences the duration of a CD8 T cell response by regulating the period of IL-2 production and possibly controlling the manifestation of AINR.

Although AINR can be induced after optimal stimulation of CD8 cells it is thought to be related to classical anergy (Deeths *et al.*, 1999). Moreover, anergy is often defined as the inability of a T cell to produce IL-2 upon restimulation

(Jenkins and Schwartz, 1987). Interestingly, anergic CD8 cells have been shown to possess elevated Fyn kinase activity (Welke and Zavazava, 2002; Utting *et al.*, 2000; Gajewski *et al.*, 1994). Also, anergised T cells lacking Fyn regain the potential to proliferate in response to antigen (Utting *et al.*, 2001). These data again support the idea that Fyn could play a role in negatively regulating the activation status of a T cell, possibly though controlling IL-2 production with relevance to the induction of anergy.

In our hands, increased IL-2 production in the absence of Fyn specifically led to improved T cell survival correlating with elevated proliferation at 72 hrs of culture, but had no measurable influence before this point. The role of IL-2 in T cell function is complex, initial studies suggested that it functioned as a mitogen driving and sustaining T cell proliferation (Morgan *et al.*, 1976). Indeed IL-2 has been shown to regulate cyclins and cell cycle progression through the induction of genes such as *c-myc, cyclin D2* and *cyclin E* (Lord *et al.*, 2000; Lord *et al.*, 1998; Liu *et al.*, 1995; Minami *et al.*, 1995). However this may not be its primary role as CD8 cells can proliferate in the absence of IL-2 (Kramer *et al.*, 1994). IL-2 has also been shown to act as a T cell survival factor by up-regulating anti-apoptotic molecules such as Bcl-2 (Lord *et al.*, 1998; Akbar *et al.*, 1996). In concordance with our data, a role for IL-2 in supporting the late stage of CD8 T cell expansion has also been shown *in vivo* by Lefrancois and colleagues. They showed that IL-2 was dispensable for initiating the response of OT-1 $Il2^{-/-}$ CD8 cells to antigen, but greatly improved the proliferative response at later periods, possibly through increasing cell survival (D'Souza and Lefrancois, 2003) This may reflect that there is a time in the expansion phase of CD8 cells where IL-2 is in limiting

quantities, so differences in production may have profound and amplified effects on T cell survival.

The increase in IL-2 levels in the absence of Fyn was also interesting as there was no evidence of increased cell death after exposure to this cytokine. While IL-2 was initially considered to be a positive regulator of T cell function, analysis of *Il2*$^{-/-}$ mice and IL-2 receptor α or β deficient mice revealed that it also plays a role in maintaining peripheral tolerance. These animals did not suffer from immunodeficiency, as may have been expected, but instead displayed severe lymphoproliferation and auto-immunity (Suzuki *et al.*, 1995; Willerford *et al.*, 1995; Sadlack *et al.*, 1993). A possible explanation for this phenotype is that IL-2 has also been shown to promote AICD by up-regulating expression of FasL (Kneitz *et al.*, 1995). However work by Cheng et al that showed enhanced IL-2 receptor signalling in CD8 cells promoted survival and proliferation but not death (Cheng *et al.*, 2002), supporting our observation that IL-2 exposure may favour survival over death in CD8 cells.

A further explanation for the lack of AICD is that Fyn has been shown to associate with the intra-cellular domain of Fas (Atkinson *et al.*, 1996), and a previous report has shown that Fyn$^{-/-}$ cells are resistant to Fas-mediated AICD (Ricci *et al.*, 2001). Although expression of FasL was not addressed in our study, the ability of F5 Fyn$^{-/-}$ to undergo Fas mediated AICD in response to anti-Fas antibody was measured (data not shown). Consistent with previous reports, F5 Fyn$^{-/-}$ cells were resistant to AICD induced by cross-linking the Fas receptor with the Jo-2 mAb.

A consequence of elevated IL-2 levels was to prolong CD25 expression on the surface of $Fyn^{-/-}$ cells. Initial CD25 expression is driven by TCR derived signals (Kim and Leonard, 2002). However expression of CD25 is then maintained by a classical feedback loop where by IL-2 receptor signals activate STAT5, which binds to the promoter of CD25 (Kim *et al.*, 2001). IL-2-driven CD25 expression is antagonised by molecules such as SOCS-1 and SOCS-3 (Sporri *et al.*, 2001; Alexander *et al.*, 1999; Cohney *et al.*, 1999; Starr *et al.*, 1997). Expression of these molecules are also regulated by IL-2 receptor signals (Sporri *et al.*, 2001). We did not see any influence of Fyn deficiency on the TCR driven CD25 expression that occured before 24 hrs. However the prolonged expression of CD25 by F5 $Fyn^{-/-}$ cells after this time could be solely attributed to increased IL-2 content in the culture well as down-modulation could be forced by the inclusion of the blocking antibody S4B6. The experiments manipulating IL-2 levels did not exclude that a loss of SOCS/CIS function contributed to prolonged CD25 expression on F5 $Fyn^{-/-}$ cells. Evidence for a possible defect in SOCS function comes from the fact that addition of excess recombinant IL-2 to F5 WT cells did not induce CD25 expression levels equivalent to those on untreated F5 $Fyn^{-/-}$ control cells. Therefore, although IL-2 levels available to the F5 WT cells would surpass even that available to untreated F5 $Fyn^{-/-}$ cells, yet expression levels of CD25 were still reduced. These observations suggest that there may be a mechanism present in WT cells that limits CD25 expression regardless of IL-2 levels, which is not functioning as efficiently in $Fyn^{-/-}$ cells. It is a possibility that IL-2 receptor signals are altered in the absence of Fyn, as studies have shown that Src kinases can associate with the IL-2 receptor β chain, and may play a role in signalling (Karnitz *et al.*, 1994). Furthermore, the region of the IL-2R β chain

containing Tyr 338, that may bind Lck, (Hatakeyama *et al.*, 1991) has been shown to link the IL-2 receptor to several pathways including MAPK (Friedmann *et al.*, 1996), PI 3-kinase (Remillard *et al.*, 1991) and STAT5 (Gaffen *et al.*, 1995).

Analysis of the periphery of the F5 $Fyn^{-/-}$ mice revealed that they were comparable to F5 WT mice in terms of cells frequency and expression of the T cell markers examined. Work by Yasuda et al showed that $Fyn^{-/-}$ cells from polyclonal mice had an a pre-activated phenotype with lower levels of CD62L expression than WT mice (Yasuda *et al.*, 2002). We did not find this to be the case in our F5 or polyclonal $Fyn^{-/-}$ mouse lines. There was however a statistically significant increase in the overall peripheral cellularity of the F5 $Fyn^{-/-}$ mice. The reason for this is unclear, but may be due to the fact that the levels of IL-7 receptor on the surface of peripheral F5 $Fyn^{-/-}$ cells were higher than F5 WT levels (data not shown). As IL-7 has been shown to regulate survival of T cells (Schluns and Lefrancois, 2003), and mice expressing IL-7 as a transgene also have increased peripheral cell numbers (Kieper *et al.*, 2002), it is possible that increased IL-7R expression may have led to prolonged naïve cell survival and, therefore, increased peripheral numbers. It is also a possibility that the increase in peripheral CD8 numbers is an *in vivo* manifestation of the hyper-responsive phenotype of the F5 $Fyn^{-/-}$ cells. This may be driven by responses to self-peptide MHC that are responsible for driving homeostatic proliferation and providing survival signals to a T cell without causing activation. Indeed, there was no evidence of an increase in CD44+ cells in the absence of Fyn (data not shown).

The F5 TCR is restricted to recognise MHC class I molecules (Townsend *et al.*, 1985; Townsend and McMichael, 1985) and has been used to study positive selection to the CD8SP lineage (Mamalaki *et al.*, 1993b; Mamalaki *et al.*, 1993a; Mamalaki *et al.*, 1992). It is clear that in the absence of Fyn, there was no overt phenotypic changes in the F5 system as the numbers and frequency of DP and CD8SP cells were comparable to F5 WT thymi, with no CD4SP evident in either group. However, when the DN populations were examined, the loss of Fyn did increase the frequency and absolute number of DN3 cells. There was also a concomittent decrease in the frequency and numbers of DN4 cells. This was not reported by Stein et al or Appleby et al, also a number of other studies have shown that Fyn is not essential to thymocyte development of αβ T cells (Lin *et al.*, 2000; Liao *et al.*, 1997). In the F5 system the mature TCR is expressed earlier than normal from the CD2 expression cassette and may override any requirement for the pre-TCR. Instead of associating with the pre-Tα, the F5 β chain will presumably associate with the transgenic alpha-chain. As there is no co-receptor expressed at the DN stage, the block in signalling may be reminiscent of the situation observed when peripheral T cells are activated through the TCR alone in the absence of Fyn. It is unclear therefore, if the partial block at the DN3 stage is a due to pre-TCR signalling or signals through the transgenic receptor itself. It is generally considered that Lck is the important kinase for pre-TCR signalling. However, it should be noted that a proportion of cells from $Lck^{-/-}$ thymi are able to undergo beta-selection and progress from the DN3 to DN4 stage (Molina *et al.*, 1992). These cells utilise Fyn because in the thymi of $Lck^{-/-}$ $Fyn^{-/-}$ mice development is completely arrested at the DN3 stage (Groves *et al.*, 1996; van Oers *et al.*, 1996b). The pre-TCR is composed of the β chain, the pre-Tα and the

CD3 complex (Groettrup *et al.*, 1993). Fyn has been shown to associate preferentially with components of the CD3 (Timson Gauen *et al.*, 1992; Samelson *et al.*, 1990); therefore it is possible that it could be involved in pre-TCR signalling in some capacity. Catalytically active Fyn expressed as a transgene does rescue some aspects of thymic development in an $Lck^{-/-}$ mouse (Groves *et al.*, 1996; Anderson *et al.*, 1992). Furthermore NK-T cell development is arrested in $Fyn^{-/-}$ mice (Eberl *et al.*, 1999; Gadue *et al.*, 1999), highlighting the fact that Fyn can influence the development of other αβ TCR expressing cell lineages. This putative role for Fyn in driving progression for DN3 to DN4 may only be evident when looking at a monoclonal TCR repertoire; therefore it may be of interest to look at the DN population in thymi from other $Fyn^{-/-}$ TCR transgenic mouse lines.

Recently, a group reported that the defects in triggering $Fyn^{-/-}$ CD4 cells could be overcome by cross-linking the CD4 co-receptor and the TCR, however they did not report whether these cells were now hyper-responsive (Sugie *et al.*, 2004). We have also addressed the role of CD4 in rescuing the proliferative response of highly purified polyclonal CD4 $Fyn^{-/-}$ cells. In our hands, activation of CD4 cells lacking Fyn was defective in response to anti-CD3 and anti- CD28 antibodies (data not shown). This was consistent with other studies that have specifically looked at $Fyn^{-/-}$ CD4 cells (Cannons *et al.*, 2004; Davidson *et al.*, 2004). However, we did not see an improvement in the response when anti-CD4 antibody was included in the stimulus (data not shown). The difference between these data and the work by Sugie et al could be explained by the fact that they looked at a monoclonal class II restricted transgenic TCR, whereas we looked at

purified naïve polyclonal CD4. Alternatively, it is possible that CD4 and CD8 cells have different dependencies for Fyn in terms of driving activation and terminating the response. We are currently back-crossing $Fyn^{-/-}$ mice onto a class II restricted transgenic TCR background to address these questions.

From the observation made in this chapter, there are a number of questions that still need to be addressed. Firstly, although these data presented here support the idea that the inclusion of co-receptor associated Lck in the stimulation of $Fyn^{-/-}$ cells is essential to generating the hyper-responsive phenotype, a role for other CD8 associated molecules cannot be excluded. One way to address this and to determine if molecules other than Lck improve the activation response of a T cell after CD8 and TCR cross-linking, the activation of F5 $Lck^{-/-}$ cells in response to anti-CD3 and anti-CD3 plus anti-CD8 could be measured. If CD8 associated Lck is the only molecule responsible for improving T cell activation by cross-linking CD8 there should be no improvement in the response by involving this receptor versus CD3 alone.

Another interesting point raised by the work presented in this chapter is the observation that F5 WT cells failed to maintain CD25 expression at the same levels compared to $Fyn^{-/-}$ cells even in the presence of excess IL-2. This suggested that there may be changes in IL-2 receptor signalling in the absence of Fyn. Firstly, it would be of interest to look at the expression of SOCS proteins in the absence of Fyn after pMHC stimulation. Furthermore it would be interesting to compare IL-2 receptor signalling in F5 WT and $Fyn^{-/-}$ cells. To control for higher levels of endogenous IL-2 in the absence of Fyn, the F5 WT and $Fyn^{-/-}$

mice could be crossed to an IL-2$^{-/-}$ background. This would allow the exposure levels of IL-2 to be controlled by addition of known quantities of exogenous cytokine and CD25 expression, proliferation and SOCS expression assayed as the readout of IL-2 signalling.

Although we did measure the contribution of CD28 co-stimulation to the Fyn phenomenon and found no influence, it was possible that other aspects of CD28 signalling not addressed by us were affecetd in Fyn$^{-/-}$cells. For example, CD28 signalling has also been shown to induce the expression of CTLA-4 (Linsley and Ledbetter, 1993). Furthermore, Lck and Fyn have been implicated in the function of CTLA-4 by mediating phosphorylation of this molecule in intra-cellular compartments (Hu *et al.*, 2001). Therefore it is possible that the expression and/or function of endogenous CTLA-4 was reduced in the absence of Fyn and this could indirectly influence IL-2 production. By adding a recombinant CTLA-4 molecule in excess we were only asking how the cells could respond to inhibition of CD28 signalling, but were not addressing how endogenous CTLA-4 production and function occurred in the absence of Fyn. To this end, we plan to look at CTLA-4 expression by activated F5 WT and Fyn$^{-/-}$ cells.

Collectively, these data presented in this chapter suggest that Fyn may act as a negative regulator of CD8 T cell activation in response to stimuli transmitted via the TCR and CD8 co-receptor. TCR stimulation alone requires Fyn for optimal signal transduction, but inclusion of CD8 and presumably associated Lck to the TCR complex circumvents requirement for Fyn in transducing activation signals. In this situation the cells become hyper-responsive due to elevated IL-2

production, possibly due to the disregulation of Lck activity caused by a failure to efficiently form the PAG/CSK/PEP complex in these cells. The subject of the next chapter of this thesis will be how we have addressed the biochemical basis for the increased IL-2 production in the absence of Fyn.

Figure 3.1: Thymic development in F5 Fyn$^{-/-}$ mice is normal and generates a periphery phenotypically comparable to F5 WT mice.

F5 WT and F5 Fyn$^{-/-}$ thymocyte development (Figures A-D) and the peripheral phenotype were examined in 5 week-old mice (as indicated). Thymocyte development was addressed by measuring the frequency (A) and absolute number (B) of DN, DP and mature SP cells compared to age matched WT controls. TCR expression is shown for CD8SP gated thymocytes. The frequency (C) of DN cells, gated as shown after staining with anti-CD4 and anti-CD8 antibodies, were analysed for expression of CD44 and CD25 to enumerate the different populations (D). Frequencies are shown on the FACS plots for relevant cell populations, these were used to calculate absolute cell numbers. A minimum of 100,000 events were collected per thymic sample (A – B). For DN analysis (C – D) a minimum of 10,000 events were collected in the DN gate. For peripheral analysis, the frequency (E) and absolute number (F) of CD8+ TCR+ cells was determined. The expression of CD62L and CD5 on TCR^{+} CD8^{+} cells is also shown (Figure E), and the frequency of cells or the mean MFI values are shown where appropriate. For peripheral analysis, a minimum of 20,000 events was collected per sample. On each graph of cell number, the mean (n = 5) is plotted and error bars are shown as ±SD. Statistical significance was calculated using the t-test (* = $p>0.05$ ** = $p>0.01$).

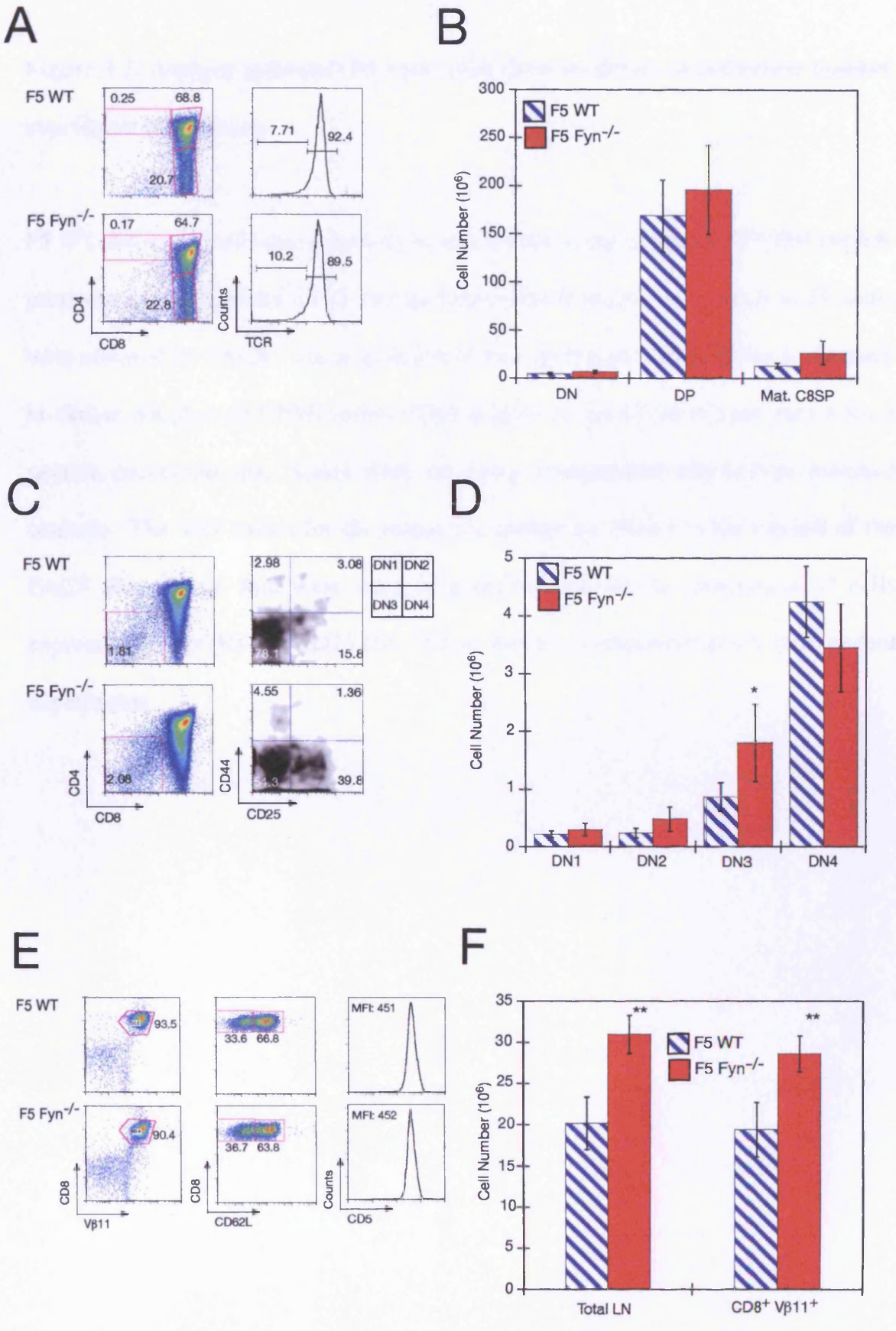

A
F5 WT
0.25
68.8
20.7
7.71
92.4
F5 Fyn-/-
0.17
64.7
22.8
10.2
89.5
CD4
CD8
Counts
TCR
B
F5 WT
F5 Fyn-/-
Cell Number (10^6)
300
250
200
150
100
50
0
DN
DP
Mat. C8SP
C
F5 WT
1.81
2.98
3.08
78.1
15.8
DN1
DN2
DN3
DN4
F5 Fyn-/-
2.08
4.55
1.36
4.3
39.8
CD4
CD8
CD44
CD25
D
F5 WT
F5 Fyn-/-
Cell Number (10^6)
5
4
3
2
1
0
*
DN1
DN2
DN3
DN4
E
F5 WT
93.5
33.6
66.8
MFI: 451
F5 Fyn-/-
90.4
36.7
63.8
MFI: 452
CD8
Vβ11
CD8
CD62L
Counts
CD5
F
Cell Number (10^6)
35
30
25
20
15
10
5
0
**
**
F5 WT
F5 Fyn-/-
Total LN
CD8+ Vβ11+

Figure 3.2: Antigen activated F5 Fyn$^{-/-}$ cells show no defect in activation marker expression at 24 hours.

F5 WT and Fyn$^{-/-}$ cells (as indicated) were activated using a titration of NP68 peptide presented by endogenous APCs and the expression of activation markers at 24 hours were assessed by FACS. As an example of how gating and analysis was performed, bi-variant dot plots of CFSE versus CD69 (Figure A and CD25 (C) are shown for 3 peptide concentrations. Gates were set using unstimulated and isotype matched controls. The MFI values for the respective marker are shown in the top left of the FACS plot. These data were used to generate plots of the percentage of cells expressing CD69 (B) and CD25 (D). These data are representative of 5 independent experiments.

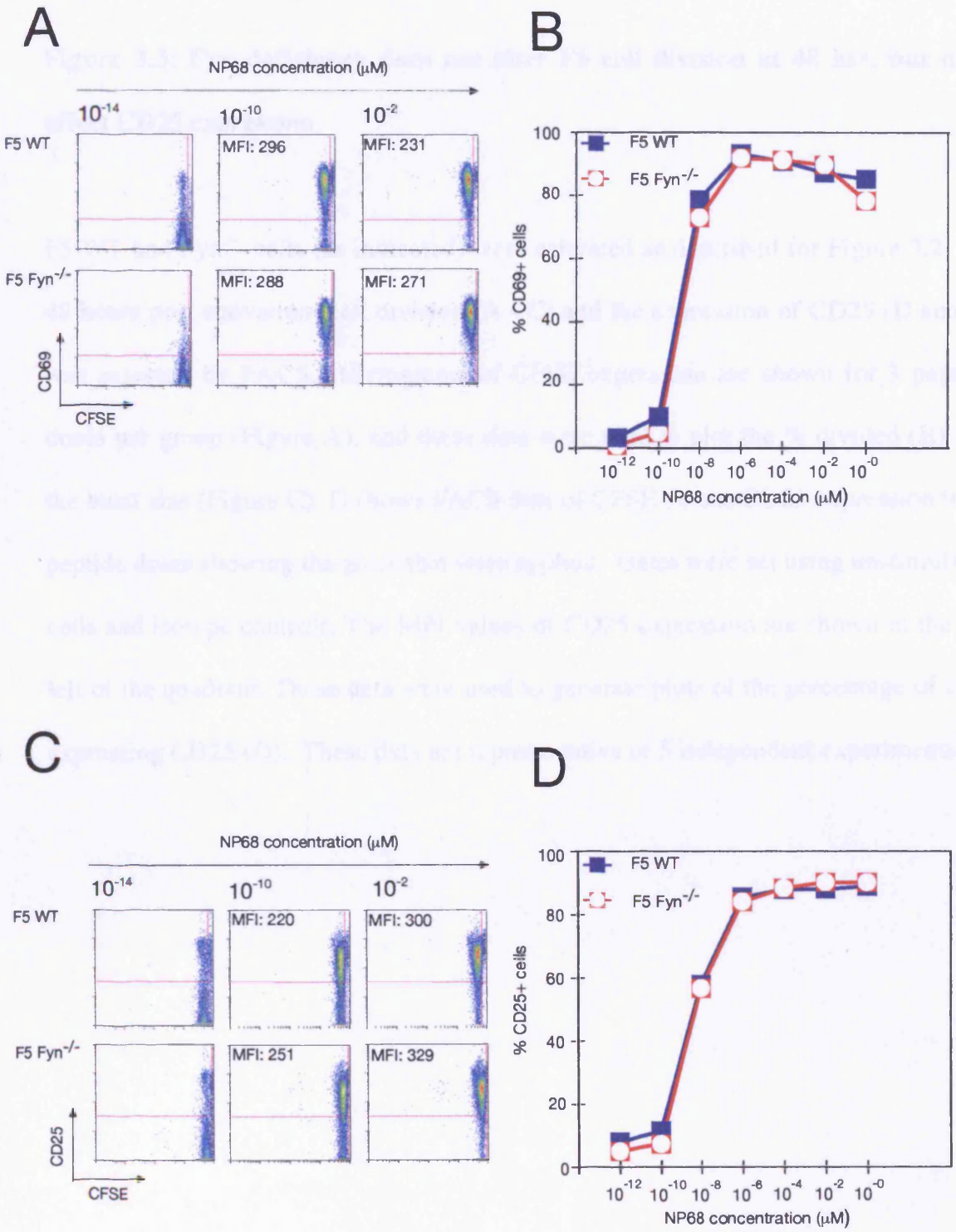
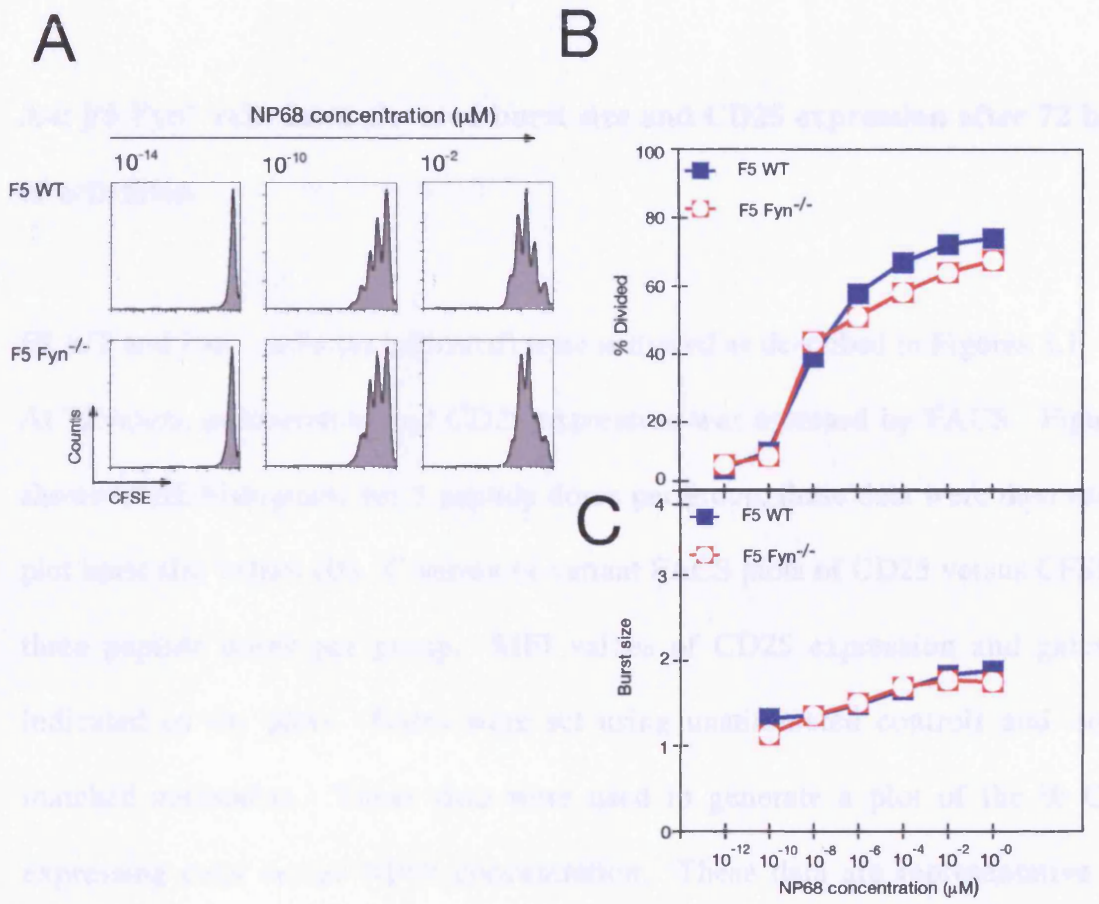
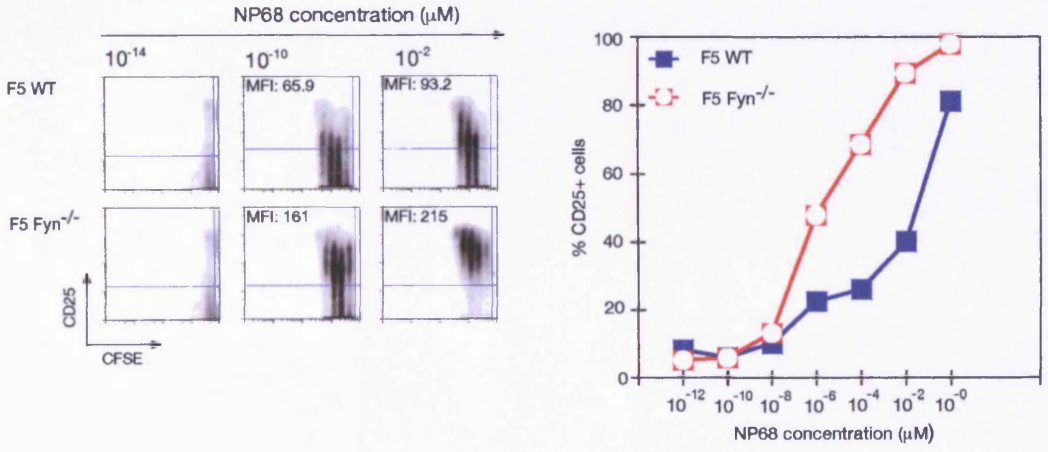

A
NP68 concentration (μM)
10-14
10-10
10-2
F5 WT
MFI: 296
MFI: 231
F5 Fyn-/-
MFI: 288
MFI: 271
CD69
CFSE
B
100
80
60
40
20
0
% CD69+ cells
F5 WT
F5 Fyn-/-
10-12 10-10 10-8 10-6 10-4 10-2 10-0
NP68 concentration (μM)
C
NP68 concentration (μM)
10-14
10-10
10-2
F5 WT
MFI: 220
MFI: 300
F5 Fyn-/-
MFI: 251
MFI: 329
CD25
CFSE
D
100
80
60
40
20
0
% CD25+ cells
F5 WT
F5 Fyn-/-
10-12 10-10 10-8 10-6 10-4 10-2 10-0
NP68 concentration (μM)

Figure 3.3: Fyn deficiency does not alter F5 cell division at 48 hrs, but does affect CD25 expression

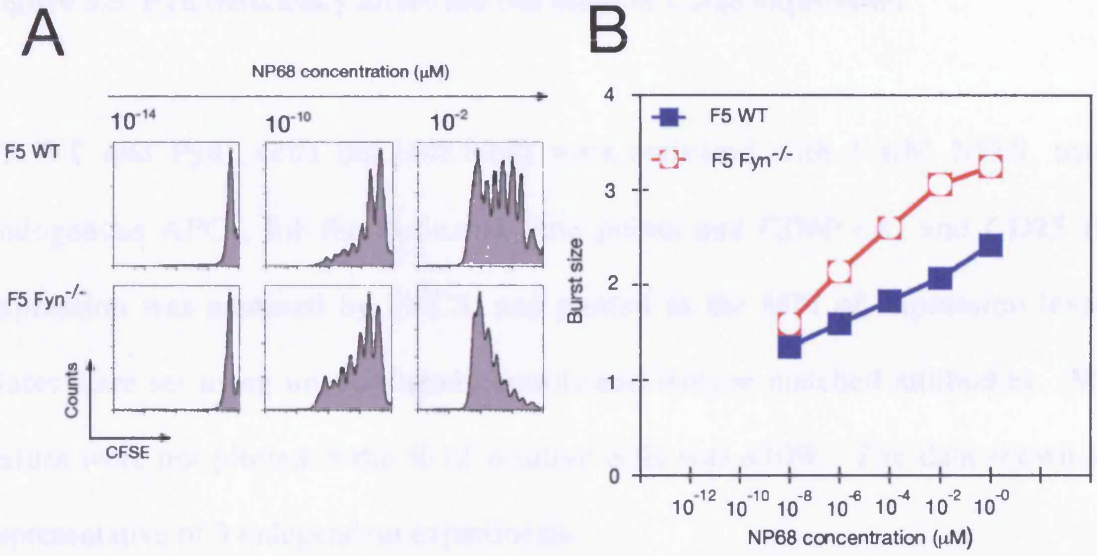
F5 WT and $Fyn^{-/-}$ cells (as indicated) were activated as described for Figure 3.2. At 48 hours post activation, cell division (A - C) and the expression of CD25 (D and E) was assessed by FACS. Histograms of CFSE expression are shown for 3 peptide doses per group (Figure A), and these data were used to plot the % divided (B) and the burst size (Figure C). D shows FACS data of CFSE versus CD25 expression for 3 peptide doses showing the gates that were applied. Gates were set using unstimulated cells and isotype controls. The MFI values of CD25 expression are shown in the top left of the quadrant. These data were used to generate plots of the percentage of cells expressing CD25 (D). These data are representative of 5 independent experiments.


A
B
C
NP68 concentration (μM)
10^{-14}
10^{-10}
10^{-2}
F5 WT
F5 Fyn$^{-/-}$
Counts
CFSE
% Divided
Burst size
10^{-12} 10^{-10} 10^{-8} 10^{-6} 10^{-4} 10^{-2} 10^{-0}
NP68 concentration (μM)


D
E
NP68 concentration (μM)
10^{-14}
10^{-10}
10^{-2}
F5 WT
F5 Fyn$^{-/-}$
MFI: 65.9
MFI: 93.2
MFI: 161
MFI: 215
CD25
CFSE
% CD25+ cells
10^{-12} 10^{-10} 10^{-8} 10^{-6} 10^{-4} 10^{-2} 10^{-0}
NP68 concentration (μM)

3.4: F5 Fyn$^{-/-}$ cells have elevated burst size and CD25 expression after 72 hours of activation

F5 WT and Fyn$^{-/-}$ cells (as indicated) were activated as described in Figures 3.1 – 3.2. At 72 hours, proliferation and CD25 expression was assessed by FACS. Figure A shows CFSE histograms for 3 peptide doses per group, these data were then used to plot burst size values (B). C shows bi-variant FACS plots of CD25 versus CFSE for three peptide doses per group. MFI values of CD25 expression and gates are indicated on the plots. Gates were set using unstimulated controls and isotype matched antibodies. These data were used to generate a plot of the % CD25 expressing cells versus NP68 concentration. These data are representative of 5 independent experiments.


A
NP68 concentration (μM)
10-14
10-10
10-2
F5 WT
F5 Fyn-/-
Counts
CFSE
B
F5 WT
F5 Fyn-/-
Burst size
0
1
2
3
4
10-12 10-10 10-8 10-6 10-4 10-2 10-0
NP68 concentration (μM)

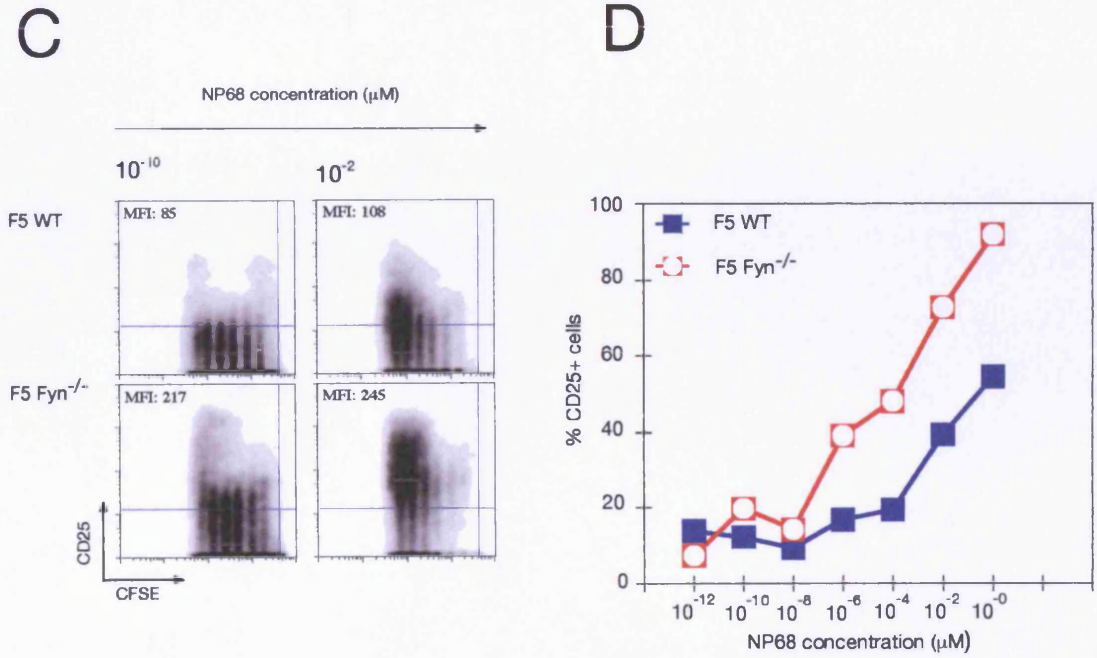
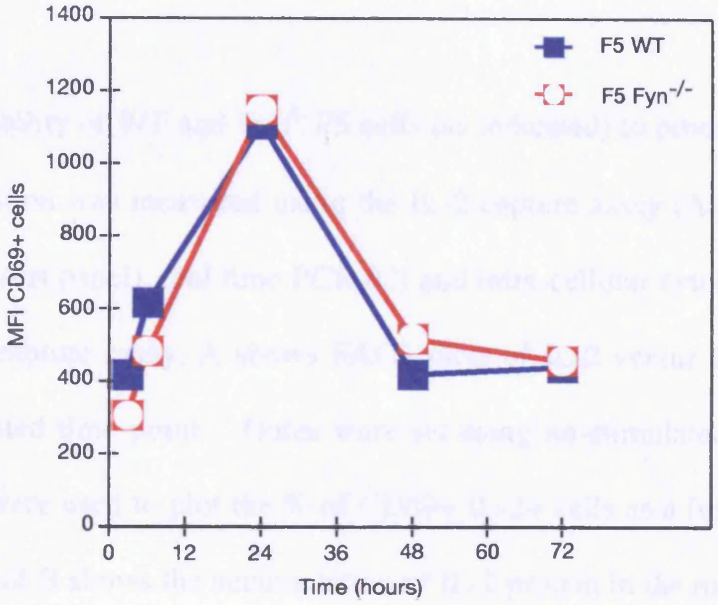

C
NP68 concentration (μM)
10-10
10-2
F5 WT
MFI: 85
MFI: 108
F5 Fyn-/-
MFI: 217
MFI: 245
CD25
CFSE
D
F5 WT
F5 Fyn-/-
% CD25+ cells
0
20
40
60
80
100
10-12 10-10 10-8 10-6 10-4 10-2 10-0
NP68 concentration (μM)

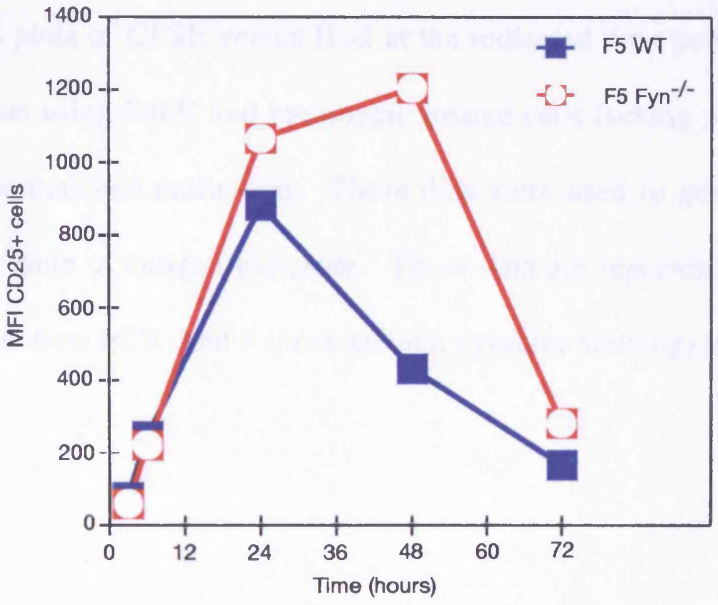
Figure 3.5: Fyn deficiency alters the duration of CD25 expression

F5 WT and $Fyn^{-/-}$ cells (as indicated) were activated with 1 μM NP68, using endogenous APCs, for the indicated time points and CD69 (A) and CD25 (B) expression was assessed by FACS, and plotted as the MFI of expression levels. Gates were set using unstimulated controls and isotype matched antibodies. MFI values were not plotted if the % of positive cells was ≤10%. The data shown are representative of 3 independent experiments.

A


1400
1200
1000
800
600
400
200
0
MFI CD69+ cells
0
12
24
36
48
60
72
Time (hours)
F5 WT
F5 Fyn-/-

B

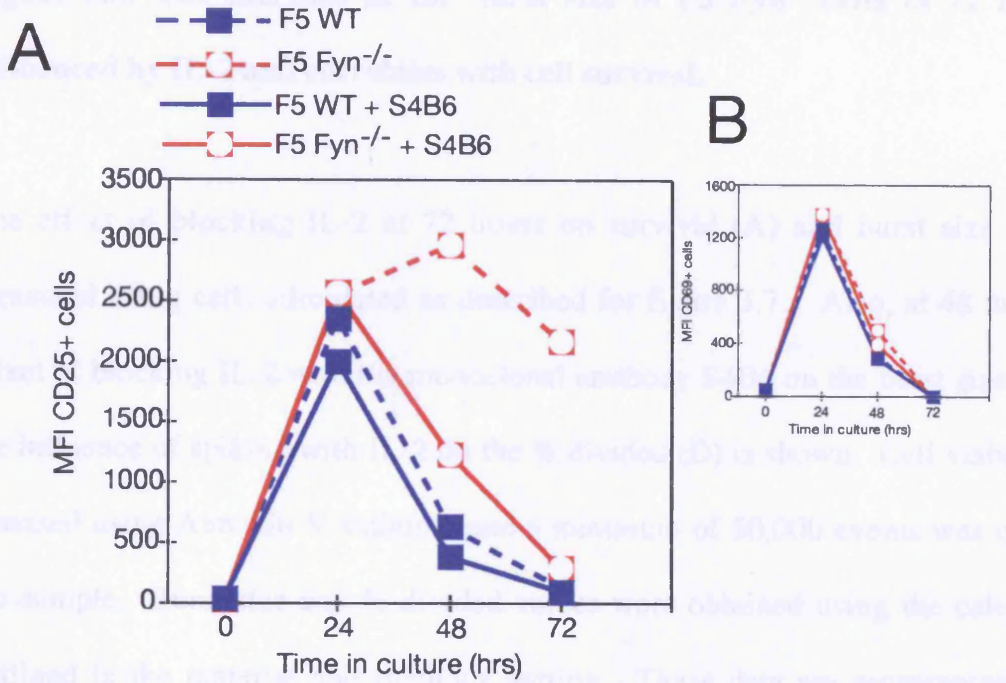

1400
1200
1000
800
600
400
200
0
MFI CD25+ cells
0
12
24
36
48
60
72
Time (hours)
F5 WT
F5 Fyn-/-

3.6: F5 Fyn$^{-/-}$ cells activated with antigen make more IL-2 than F5 WT cells

The ability of WT and Fyn$^{-/-}$ F5 cells (as indicated) to produce IL-2 after 1 μM NP68 activation was measured using the IL-2 capture assay (A and B), CTLL-2 bioassay (B, insert panel), real time PCR (C) and intra-cellular cytokine staining (D). For the IL-2 capture assay, A shows FACS plots of IL-2 versus CD69 expression for each indicated time point. Gates were set using un-stimulated control samples. These data were used to plot the % of CD69+ IL-2+ cells as a function of time. The insert panel of B shows the accumulation of IL-2 protein in the media at 48 hours measured by the CTLL-2 bioassay. For real time PCR analysis, RNA was extracted from 1 x 10^6 cells at the indicated time points. After cDNA was prepared, real time PCR for IL-2 mRNA was carried out . The values plotted in C show IL-2 mRNA levels relative to the housekeeping gene HPRT. For intracellular detection of IL-2 D shows FACS plots of CFSE versus IL-2 at the indicated time points for each group. Gates were set using PdbU and ionomycin treated cells lacking prior pMHC exposure, and isotype matched antibodies. These data were used to generate a plot of IL-2 MFI versus time of antigen exposure. These data are representative of 2 (Capture assay and real time PCR) and 3 (intra-cellular cytokine staining) independent experiments.

Figure 3.7: Prolonged CD25 expression by F5 Fyn$^{-/-}$ cells is dependent on IL-2

The IL-2 availability for antigen activated WT and Fyn$^{-/-}$ cells was manipulated as indicated in the graph legends. The effect that blocking IL-2 with S4B6 had on the MFI of CD25 expression (A) and CD69 expression (B) is shown. Figures C and D show the effect that spiking with 50 U of recombinant IL-2 had on the MFI of CD25 (B) and CD69 (D) expression respectively. Gates were set using unstimulated cells and isotype controls. MFI values were only plotted when the % of cells expressing an activation marker was ≤10%. These data are representative of 2 independent experiments.


A
F5 WT
F5 Fyn-/-
F5 WT + S4B6
F5 Fyn-/- + S4B6
MFI CD25+ cells
3500
3000
2500
2000
1500
1000
500
0
0
24
48
72
Time in culture (hrs)
B
MFI CD69+ cells
1600
1200
800
400
0
0
24
48
72
Time in culture (hrs)

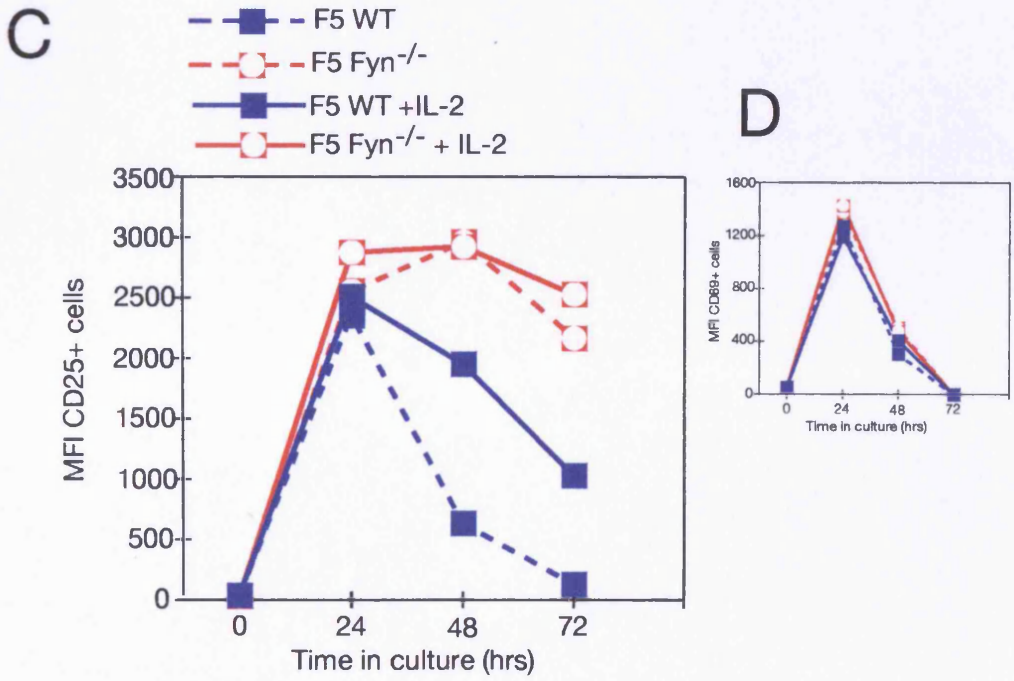

C
F5 WT
F5 Fyn-/-
F5 WT +IL-2
F5 Fyn-/- + IL-2
MFI CD25+ cells
3500
3000
2500
2000
1500
1000
500
0
0
24
48
72
Time in culture (hrs)
D
MFI CD69+ cells
1600
1200
800
400
0
0
24
48
72
Time in culture (hrs)

Figure 3.8: The increase in the burst size of F5 Fyn$^{-/-}$ cells at 72 hours is influenced by IL-2 and correlates with cell survival.

The effect of blocking IL-2 at 72 hours on survival (A) and burst size (B) was measured using cells stimulated as described for figure 3.7. Also, at 48 hours, the effect of blocking IL-2 with the monoclonal antibody S4B6 on the burst size (C) and the influence of spiking with IL-2 on the % divided (D) is shown. Cell viability was assessed using Annexin V staining, and a minimum of 50,000 events was collected per-sample. Burst size and % divided values were obtained using the calculations outlined in the material and methods section. These data are representative of 2 independent experiments.

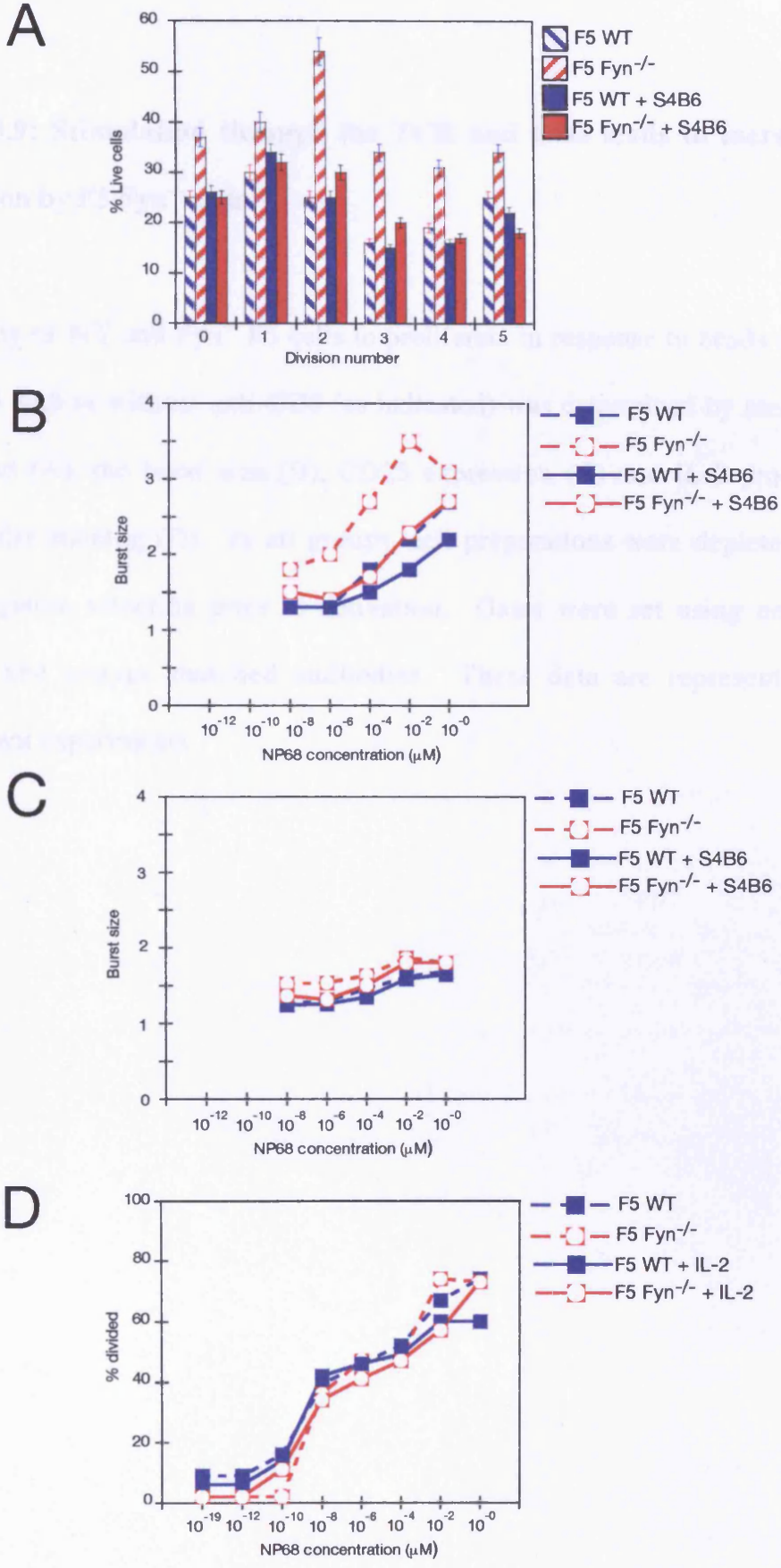

A
% Live cells
Division number
F5 WT
F5 Fyn-/-
F5 WT + S4B6
F5 Fyn-/- + S4B6
B
Burst size
NP68 concentration (μM)
F5 WT
F5 Fyn-/-
F5 WT + S4B6
F5 Fyn-/- + S4B6
C
Burst size
NP68 concentration (μM)
F5 WT
F5 Fyn-/-
F5 WT + S4B6
F5 Fyn-/- + S4B6
D
% divided
NP68 concentration (μM)
F5 WT
F5 Fyn-/-
F5 WT + IL-2
F5 Fyn-/- + IL-2

Figure 3.9: Stimulation through the TCR and CD8 leads to increased IL-2 production by F5 Fyn$^{-/-}$ cells.

The ability of WT and Fyn$^{-/-}$ F5 cells to proliferate in response to beads coated with anti-CD3 with or without anti-CD8 (as indicated) was determined by measuring the % divided (A), the burst size (B), CD25 expression (C) and IL-2 production by intracellular staining (D). In all groups, cell preparations were depleted of APCs using negative selection prior to activation. Gates were set using unstimulated controls and isotype matched antibodies. These data are representative of 2 independent experiments.

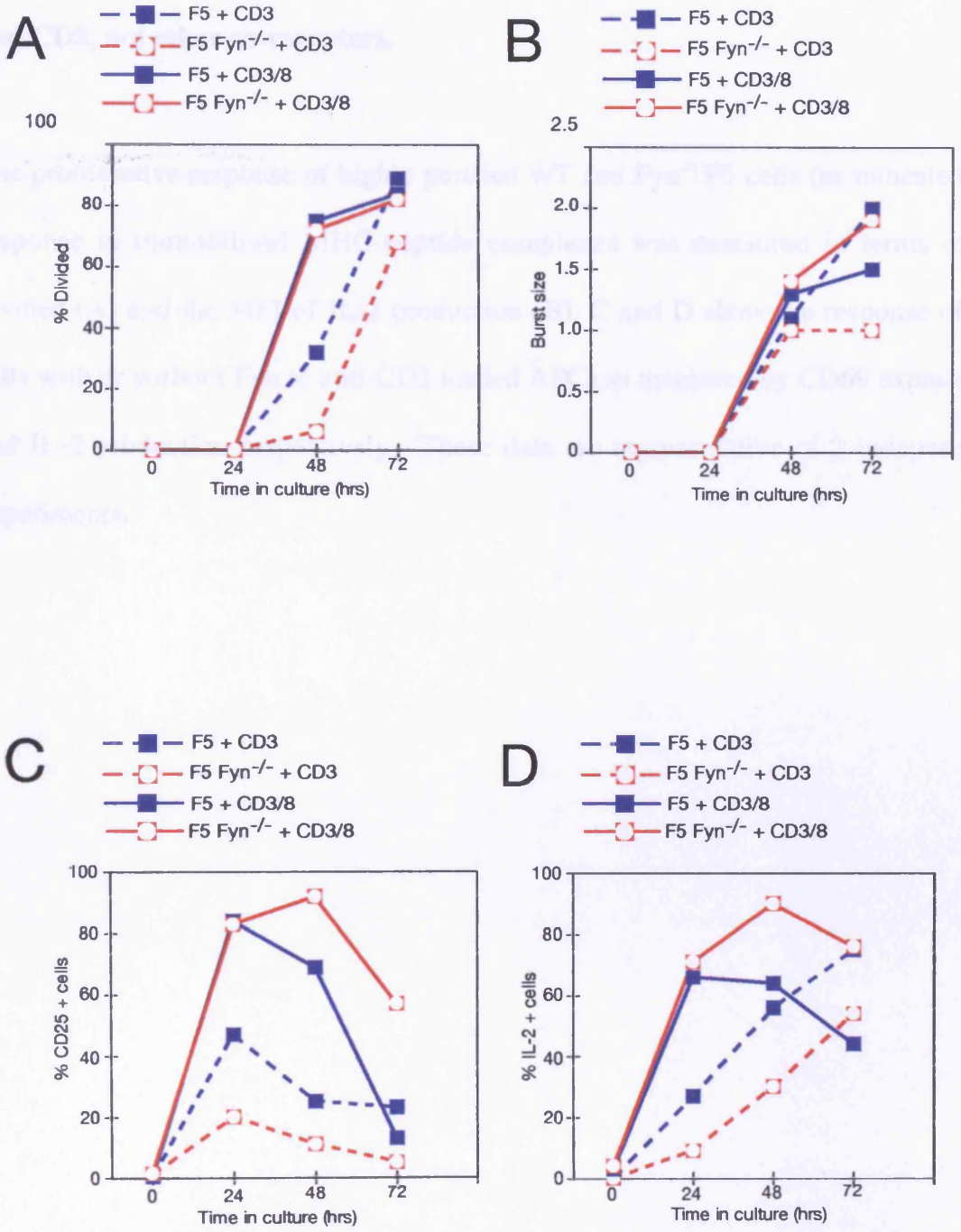

A
F5 + CD3
F5 Fyn-/- + CD3
F5 + CD3/8
F5 Fyn-/- + CD3/8
% Divided
Time in culture (hrs)
B
Burst size
Time in culture (hrs)
C
% CD25 + cells
Time in culture (hrs)
D
% IL-2 + cells
Time in culture (hrs)

Figure 3.10: Elevated IL-2 production by F5 Fyn$^{-/-}$ cells is regulated by the TCR and CD8, not other co-receptors.

The proliferative response of highly purified WT and Fyn$^{-/-}$ F5 cells (as indicated) in response to immobilised MHC-peptide complexes was measured in terms of % divided (A) and the MFI of IL-2 production (B). C and D show the response of F5 cells with or without Fyn to anti-CD3 loaded APCs as measured by CD69 expression and IL-2 production respectively. These data are representative of 2 independent experiments.

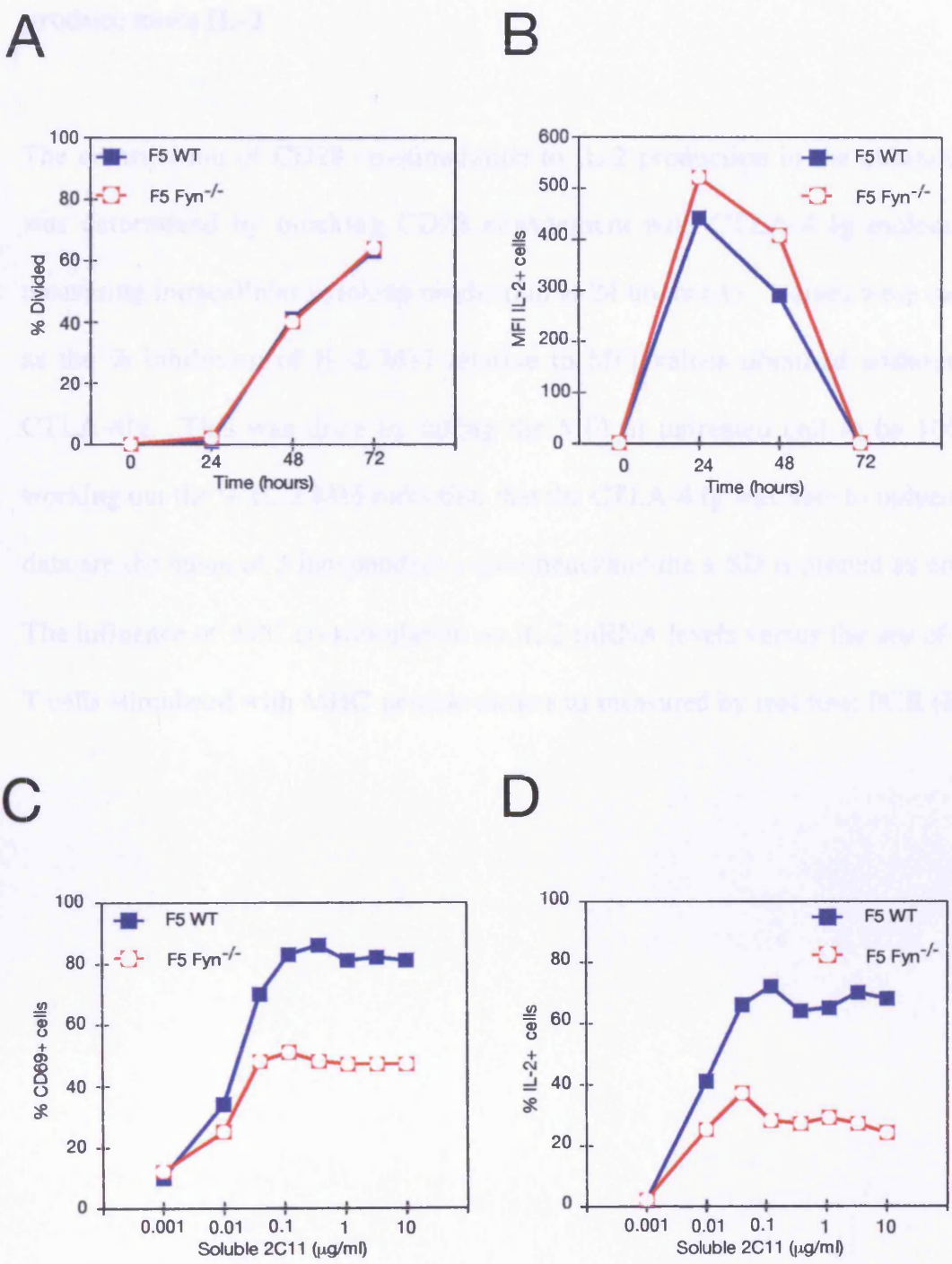
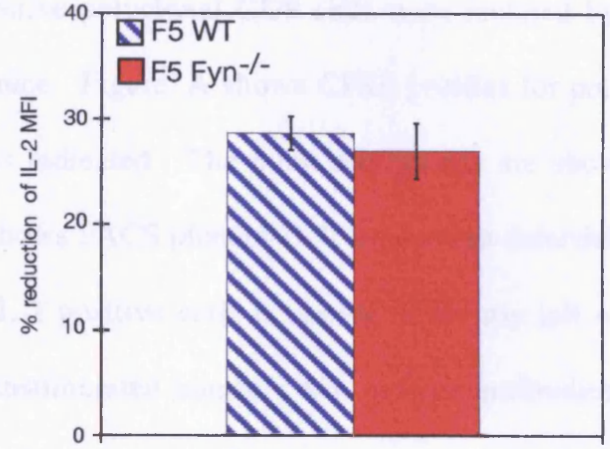

A
B
C
D
F5 WT
F5 Fyn-/-
% Divided
Time (hours)
MFI IL-2+ cells
% CD69+ cells
% IL-2+ cells
Soluble 2C11 (μg/ml)
0
20
40
60
80
100
200
300
400
500
600
24
48
72
0.001
0.01
0.1
1
10

Figure 3.11: CD28 co-stimulation does not control the ability of F5 Fyn$^{-/-}$ cells to produce more IL-2

The contribution of CD28 co-stimulation to IL-2 production in the absence of Fyn was determined by blocking CD28 engagement with CTLA-4 Ig molecules and measuring intracellular cytokine production at 24 hours (A). Values were calculated as the % inhibition of IL-2 MFI relative to MFI values obtained without adding CTLA-4Ig. This was done by taking the MFI of untreated cell to be 100%, and working out the % IL-2 MFI reduction that the CTLA-4 Ig was able to induce. These data are the mean of 3 independent experiments and the ± SD is plotted as error bars. The influence of APC co-stimulation on IL-2 mRNA levels versus the use of purified T cells stimulated with MHC-peptide dimers as measured by real time PCR (B).

A


F5 WT
F5 Fyn-/-
% reduction of IL-2 MFI
40
30
20
10
0

B

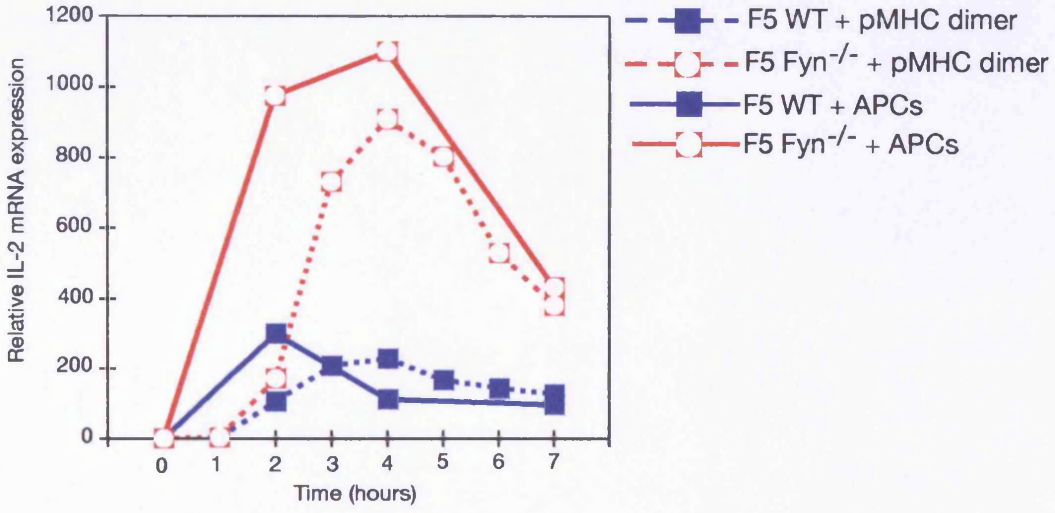
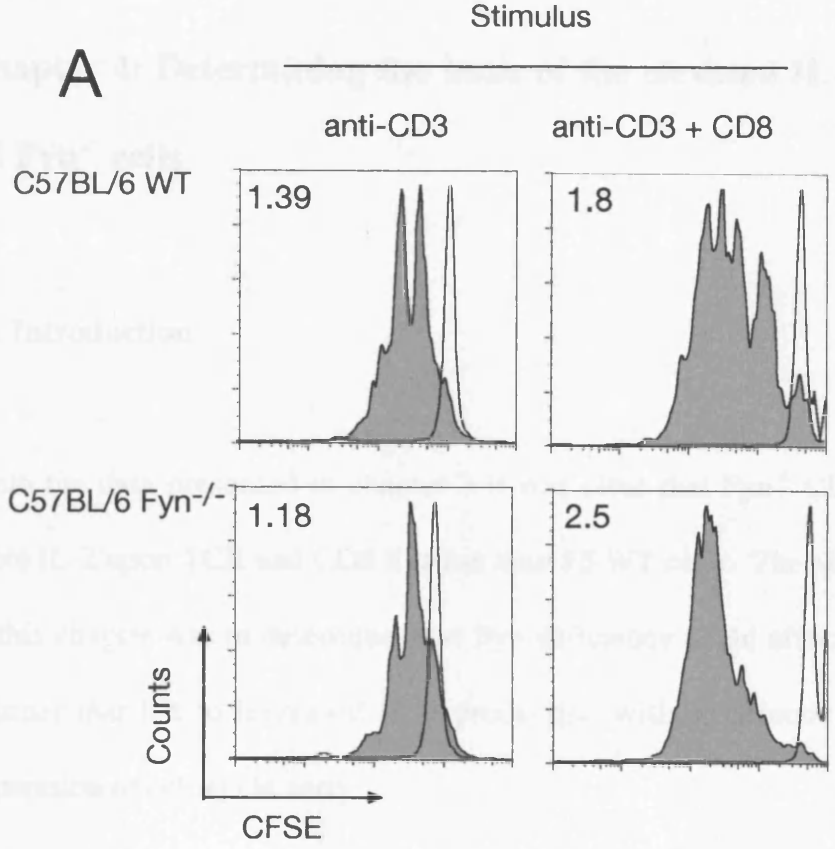
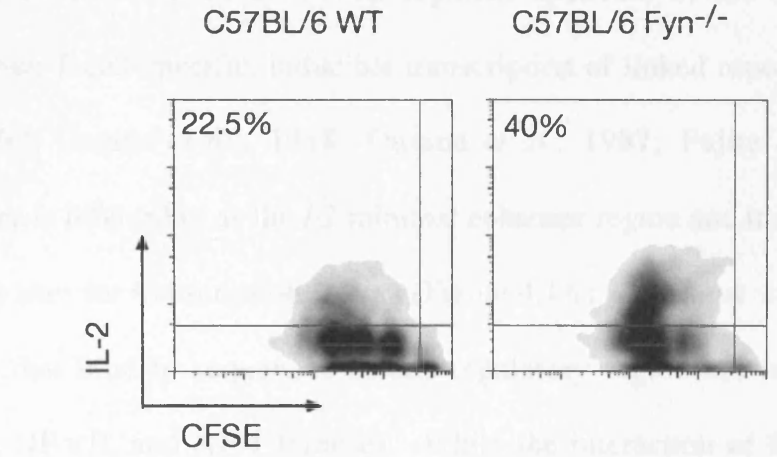

F5 WT + pMHC dimer
F5 Fyn-/- + pMHC dimer
F5 WT + APCs
F5 Fyn-/- + APCs
Relative IL-2 mRNA expression
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
Time (hours)

Figure 3.12: Crosslinking TCR and CD8 molecules on polyclonal CD8 $Fyn^{-/-}$ induces elevated burst size and IL-2 production at 24 hrs

Naïve polyclonal CD8 cells were isolated by FACS from the LN of WT or $Fyn^{-/-}$ mice. Figure A shows CFSE profiles for polyclonal WT and $Fyn^{-/-}$ cells stimulated as indicated. The burst size values are shown in the top left of the histogram. B shows FACS plots of IL-2 production detected by intra-cellular staining and the % of IL-2 positive cells is shown in the top left of the quadrant. Gates were set using unstimulated controls and isotype antibodies. These data are representative of 2 independent experiments.


Stimulus
A
anti-CD3
anti-CD3 + CD8
C57BL/6 WT
1.39
1.8
C57BL/6 Fyn-/-
1.18
2.5
Counts
CFSE


B
C57BL/6 WT
C57BL/6 Fyn-/-
22.5%
40%
IL-2
CFSE

Chapter 4: Determining the basis of the elevated IL-2 production by F5 Fyn$^{-/-}$ cells

4.1 Introduction

From the data presented in chapter 3 it was clear that Fyn$^{-/-}$ CD8 T cells produced more IL-2 upon TCR and CD8 ligation than F5 WT cells. The aim of work presented in this chapter was to determine how Fyn deficiency could affect T cell signalling in manner that led to increased IL-2 production without affecting activation marker expression or cell cycle entry.

Regulation of the *Il2* gene is complex involving many transcription factors, the activity of which, are regulated in T cells via shared and distinct signalling pathways. Studies have identified a ~300bp segment upstream of the *Il2* gene capable of conferring T cell specific, inducible transcription of linked reporter genes (Riegel *et al.*, 1992; Durand *et al.*, 1988; Durand *et al.*, 1987; Fujita *et al.*, 1986). This sequence is referred to as the *Il2* minimal enhancer region and it contains a number of binding sites for transcription factors (Figure 4.1A). The most well studied groups of factors that bind to sequences in this regulatory region are members of the Oct, NFAT, NF-κB, and AP-1 families. While the interaction of Oct-1 with its target sequence is activation independent, the binding of NFAT, NF-κB and AP-1 is induced only after T cell stimulation (Rooney *et al.*, 1995; Serfling *et al.*, 1989; Brunvand *et al.*, 1988). It is interesting that none of these factors are specific to the

Il2 gene or to T cells, and yet production of IL-2 is thought to be highly cell-type restricted and dependent upon activation. To date no single transcription factor has been identified as a unique regulator of the *Il2* gene. Instead, transcription of IL-2 seems to be achieved by the co-operative actions of all these ubiquitous factors binding to their target sequences within the minimal enhancer. Loss of binding mutations in any site has profound effects on the transcription of linked reporter constructs (Zhang and Nabel, 1994; Jain *et al.*, 1992; Thompson *et al.*, 1992; Kamps *et al.*, 1990). Interestingly, the binding sites for all these factors are non-consensus sites, possessing a ~10-fold lower affinity than corresponding consensus sites located in other genes also regulated by these common factors. This relatively weak affinity of the target sites is thought to be what controls the T cell specific production of IL-2, as conversion of these sequences from non-consensus to consensus sites is able to then drive *IL2* reporter gene transcription in non-T cells (Hentsch *et al.*, 1992). Therefore, production of IL-2 by an activated T cell is a tightly regulated process because only the correct stimulus, able to induce the co-ordinate activation and binding of all factors to their relatively weak recognition sites, will induce transcription of this cytokine. In contrast partial or sub-optimal stimuli may lead only to the activation of one or two factors and will not be sufficient to drive IL-2 production. Certainly, DNA foot-print analysis has revealed that all sites in the *Il2* minimal enhancer are occupied when the factors are present and active, or all empty if only a few are functional (Chen and Rothenberg, 1994; Garrity *et al.*, 1994).

The factors that bind to the *Il2* minimal enhancer region can be sub-divided into two categories depending on when they can be detected in the nucleus (Jain *et al.*, 1995) (Figure 4.1B). The earliest factors present within 1 hour following activation include NFATc2, AP-1, and Rel-A. AP-1 and Rel-A, in concert with other transcription factors activated within this time frame (≤1 hour) have been shown to induce the expression of CD69 (Castellanos *et al.*, 1997) and CD25 (John *et al.*, 1995) respectively. However IL-2 transcription also requires the action of late factors such as c-Rel, Oct-2 and NFATc1 that are detectable in the nucleus ~4 hrs post activation (Venkataraman *et al.*, 1995; Northrop *et al.*, 1994; Kang *et al.*, 1992). The need for these distal factors correlates with the observation that IL-2 production requires a more prolonged period of stimulation than the expression of activation markers. For example stimulation with soluble antibodies is capable of inducing expression of CD69 but not IL-2 (Wacholtz and Lipsky, 1993). With such a stimulus, the signals will be rapidly terminated because the antibodies will be internalised. In contrast, antibodies immobilised on a fixed surface will not be internalised inducing more sustained signals resulting in IL-2 production. Also treatment of Con A activated T cells with the competitive inhibitor alpha-methyl mannoside showed that 2-4 hrs of sustained activation was required for IL-2 mRNA production (Weiss *et al.*, 1987). This requirement for sustained antigen exposure has also been demonstrated by several other groups by controlling the period a T cell was in contact with the stimulus and looking at the ability to express activation markers and IL-2 (Lanzavecchia *et al.*, 1999; Iezzi *et al.*, 1998; Waldrop *et al.*, 1998; Itoh and Germain, 1997; Viola and Lanzavecchia, 1996; Shaw *et al.*, 1988) or by addressing the period

of APC contact required to induce IL-2 production at the single cell level (Hurez *et al.*, 2003).

In this model (Figure 4.1B), activation marker expression is controlled by the first threshold of signal that is sufficient to activate the early factors NFATc2, Rel-A and AP-1. However IL-2 production is then regulated by a second threshold of signal, achieved only when the duration and / or intensity of signal is sufficient to activate the later factors such as c-Rel and Oct. There is one excellent piece of evidence for this theory that has been described by several groups. Within a population of activated T cells, optimal stimulations *in vitro* using immobilised antibodies or pMHC, is capable of inducing ~90% of cells to express CD69/25, however IL-2 production within this same population rarely exceeds 50% (La Gruta *et al.*, 2004; Kristensen *et al.*, 2002; Panus *et al.*, 2000; Veiga-Fernandes *et al.*, 2000; Khoruts *et al.*, 1998; Weaver *et al.*, 1998; Itoh and Germain, 1997; Rogers *et al.*, 1997; Bucy *et al.*, 1995). These data suggest that, within a population of polyclonal, and even monoclonal T cells, there is heterogeneity based on the ability of a cell to make IL-2. Therefore ~90% of cells receive sufficient signal duration/intensity to cross the threshold for activation marker expression, however only a fraction of these cells then receive the additional period of stimulation to cross the second threshold and produce IL-2 (see Figure 4.1B).

From chapter 3 it was clear that the loss of Fyn was able to increase the percentage of IL-2 producers within a population of activated cells, without altering the expression

of activation markers. These data suggested that the loss of Fyn was somehow specifically affecting the ability of cells to cross the second signalling threshold and commit to IL-2 production. Fyn deficiency could affect the threshold controlling IL-2 production in several ways. Firstly, the loss of Fyn may be affecting the function of the early factors in someway so that when the late factors became activated they would collectively result in improved efficiency of IL-2 production. Secondly, the early transcription factors may be unaffected, and instead the efficiency of the activation of the late factors may be elevated, resulting again in improved ability to produce IL-2. Thirdly, Fyn deficiency may be affecting both the early and late factors, resulting in a synergistic effect on IL-2 transcription. A further degree of complexity is introduced by considering how Fyn could be affecting these factors. For example, the effect on IL-2 production may be directly due to the loss of activation of Fyn specific substrates. Certainly, studies in human T cells revealed that Fyn may be part of a multi-protein complex consisting of basely phosphorylated proteins that include ADAP, SKAP55 and Pyk2 (Marie-Cardine *et al.*, 1999). Furthermore, upon T cell stimulation and in transformed cell lines, the composition of this complex alters, suggesting that it may play a role in regulating the activation status of the T cell (Marie-Cardine *et al.*, 1999). However, the influence on the magnitude of IL-2 production in the absence of Fyn may be more indirect, and could be mediated through the putative disregulation of Lck activity caused by a reduction of PAG phosphorylation discussed in chapter 3. Again, it cannot be discounted that a combination of these effects are contributing to the increase in IL-2 production without Fyn.

4.2 Fyn deficiency increases the frequency of activated cells able to make IL-2

To address whether we could define these putative signalling thresholds for the regulation of activation marker expression versus IL-2 production in F5 T cells, we set up experiments to measure the kinetics of CD25 and CD69 expression and IL-2 production within the same population of cells. If there were indeed a requirement for more sustained periods of activation to induce IL-2 expression compared to activation markers, we would expect observe the following. Firstly, CD69 and CD25 expression should precede IL-2 production, and secondly that not all activated cells would be able to produce IL-2. To this end F5 WT and $Fyn^{-/-}$ cells were activated using peptide loaded dimer molecules and the kinetics of CD69 and CD25 expression and IL-2 secretion were measured within the same population on a single cell basis. Figure 4.2 shows that CD69 was the earliest marker to be expressed by activated F5 cells appearing after 1 hour of activation. The timing and level of CD69 expression was comparable between the WT and $Fyn^{-/-}$ cells, reaching a plateau by 3 hrs. CD25 expression appeared at ~3 hrs in both groups, plateau at ~96% after 12 hrs. In contrast to activation marker expression, IL-2 production within these cell populations was first detected by capture assay at 4 hrs. Furthermore, this was restricted to only a sub-population of activated cells in both groups. By 5 hrs the percentage of activated F5 $Fyn^{-/-}$ cells producing IL-2 exceeded that of activated F5 WT cells, but CD69/25 expression was unaltered. Collectively these data confirmed that IL-2 production occurs after activation markers expression, and is restricted to only a sub-set of responding cells. Moreover, Fyn deficiency did not affect whether a

given cell reached the first threshold controlling activation marker expression, but it did increase the frequency of cells within a population able to reach the second threshold for IL-2 production.

4.3 The period of stimulation required to commit a cell to IL-2 production is reduced in the absence of Fyn

Having established that IL-2 production by antigen activated F5 cells occurred after the detection of activation markers, and was restricted to a subset of cells, we wanted to ask about the duration of antigen exposure that was required to induce IL-2 production versus CD69 expression. More specifically, the question was how Fyn deficiency could have affected these thresholds so that F5 $Fyn^{-/-}$ cells produced more IL-2 while not affecting up-regulation of CD69. To this end we exposed purified F5 WT and $Fyn^{-/-}$ T cells to a layer of NP68 pMHC dimers for defined periods, then removed them from the stimulus and put them into culture away from antigen and assayed these cells at 24 hrs for the expression of CD69 and intracellular IL-2 staining.

Previous studies have also addressed how long a stimulus was required for T cell activation and IL-2 production, using plate bound antibodies and pMHC-complexes (Iezzi *et al.*, 1998). However these studies did not control for the possibility of antigenic carry-over after cells were removed from the stimulus. This was a concern, as any residual antigen carried over would have compromised the interpretation of the

data. Therefore to control for this we removed the F5 cells from the stimulus, washed them extensively and placed them in wells that contained CFSE labelled naïve F5 cells that would act as indicators, becoming activated if antigen were present. Figures 4.3A and B show that there was no significant antigenic carry over in all wells, as CFSE labelled cells showed only background staining levels (CD69 ≤5% Figure 4.3A and IL-2 ≤8% Figure 4.3B). Expression of CD69 and IL-2 was then assessed after a total of 24 hrs of culture to address the level of activation and commitment to IL-2 production induced after the defined periods of antigenic contact.

From Figure 4.3C it is evident that ~3 hrs of exposure to stimulus was required to upregulate CD69 expression in 50% of cells in both groups. In contrast commitment to express IL-2 in a similar proportion of cells required a longer period of stimulation. These data supported published observations that short periods of antigen exposure were enough to induce CD69 expression but that increased periods were required for IL-2 production (Wacholtz and Lipsky, 1993; Weiss *et al.*, 1987). Remarkably, F5 $Fyn^{-/-}$ cells required a period of ~10 hrs of antigenic exposure for 50% of cells to produce IL-2, whereas F5 WT cells required ~24 hrs of antigen exposure to reach a comparable percentage of IL-2 + cells. These data indicate that Fyn deficiency reduced the period of antigenic exposure required for IL-2 production by ~2-fold compared to WT cells without affecting CD69 expression.

It is also of note that regardless of how long the cells were exposed to antigen, production of IL-2 by F5 $Fyn^{-/-}$ cells was consistently elevated compared to F5 WT cells. As increased IL-2 production by F5 $Fyn^{-/-}$ cells was seen regardless of the length of exposure to antigen, it suggested that this phenomenon did not require a particular length of time on the dimer layer. However, as the increased IL-2 production was observed after all periods of stimulation capable of inducing IL-2 in either group, it suggested that it was not the duration of signal that controlled the elevated IL-2 production but altered sensitivity of the F5 $Fyn^{-/-}$ cells.

Another interesting observation from these data is that 1 hour of stimulation was enough to induce detectable IL-2 production in the F5 $Fyn^{-/-}$ cells 24 hrs later. This was unexpected as transcription of the late factors, such as c-Rel or Oct2, should not have occurred in this 1 hour time frame of stimulation (Venkataraman *et al.*, 1995; Kang *et al.*, 1992). Indeed, WT F5 cells required at least 4 hrs of antigenic exposure to commit to IL-2 production 24 hrs later, supporting the idea that more prolonged signals are required compared to activation marker expression.

4.4 The relationship between Src kinase activity and antigen exposure is uncoupled with respect to IL-2 production in F5 $Fyn^{-/-}$ cells

From the data in Figure 4.3. There were two possible explanations for why IL-2 production could be observed after just 1 hour of antigen exposure. Firstly, in the absence of Fyn the duration of signal continued even after removal of the antigen, or

secondly that the strength of signal induced in 1 hour was elevated compared to WT leading to the premature activation of the late factors required for IL-2 transcription.

To try and resolve the reason why F5 $Fyn^{-/-}$ cells were able to make IL-2 24 hrs later after just 1 hour of antigen exposure, we decided to ask if signalling was terminated by addition of inhibitors at this time, would the signals already generated still be sufficient to drive IL-2 production 24 hrs later. We used the Src specific inhibitor PP2, adding it to F5 cells 3 hrs after triggering with peptide, and at 24 hrs we measured the ability to produce IL-2 by intracellular staining. If the signals established during the 1 hour of antigen exposure in the absence of Fyn were sufficient to induce IL-2 production, the termination of Src kinase activity at 3 hrs should not have any effect on the ability to observe IL-2 24 hrs later. The decision to add PP2 at 3 hrs and not earlier was based on the observation that CD69 expression could be achieved prior to this point, but CD25 expression could not (Figure 4.2). Therefore measuring CD69 expression before 3 hrs would indicate that cells had been successfully activated, while lack of CD25 expression after PP2 was added, would indicate that inhibition had occurred.

From Figure 4.4A it is clear that treatment of both groups with PP2 from the start of activation inhibited CD69 expression. This was specific for PP2 as the non-inhibitory, related compound, PP3, gave identical CD69 expression profile as untreated cells. Treatment of the cells with PP2 3 hrs after activation had no effect on CD69 expression in either group. When CD25 expression was measured (Figure

4.4B), again the addition of PP2 from the start of activation inhibited both groups, as did addition of PP2 after 3 hrs. These data indicate that Src kinase activity for longer than 3 hrs of antigen exposure was required to drive CD25 expression in activated CD8 T cells.

As a further measure of cell activation, we determined the level of antigen-induced TCR down-modulation. From Figure 4.4C it is clear that in both F5 WT and $Fyn^{-/-}$ treatment with PP2 from the start of activation completely blocked TCR down-modulation. TCR down-modulation occurred with the uninhibited cells and PP3 treated cells. When PP2 was added after 3 hrs of stimulation, any further TCR downmodulation was prevented and levels even increased in both groups of cells. Interestingly F5 $Fyn^{-/-}$ cells down-modulated their TCR levels more rapidly compared to WT cells in the absence of inhibitor. This may reflect a heightened degree of activation in the absence of Fyn as it is thought that TCR downmodulation occurs in response to T cell activation (Alcover and Alarcon, 2000).

When IL-2 production was measured in these cells at 24 hrs, treatment with PP2 from the start of antigen treatment eliminated IL-2 production in both groups (Figure 4.4D). This was expected, as these cells did not activate as judged by CD69 and CD25 expression. However, when PP2 was added at 3 hrs, although CD69 expression was unaffected, IL-2 production was inhibited in both groups. This was surprising, as the data in Figure 4.3 indicated that 1 hour of antigen exposure was enough to commit $Fyn^{-/-}$ cells to IL-2 production 24 hrs later, therefore one may have

expected that inhibiting Src kinase signals at 3 hrs would have had no effect in the absence of Fyn. Collectively these data, and the data in Figure 4.3, show that IL-2 production by $Fyn^{-/-}$ cells may require only 1 hour of antigen exposure, but did require Src kinase activity beyond 3 hrs.

4.5 The efficiency of conjugate formation is normal in the absence of Fyn

A possible explanation for the elevated IL-2 production in the absence of Fyn was that the formation of stable T cell APC conjugates was altered, leading to increased signal duration, which was able to push more cells over the second activation threshold (Fig 4.1B). A recent study by Weaver and colleagues has shown that T cells that form stable conjugates with an APC may be more likely to produce IL-2 (Hurez *et al.*, 2003). Conjugate formation is regulated, in part, by the re-arrangement of cytoskeletal components (Fuller *et al.*, 2003). A number of Fyn substrates such as ADAP (Wang *et al.*, 2004; Wang *et al.*, 2003; Liu *et al.*, 1998), Vav (Huang *et al.*, 2000; Michel *et al.*, 1998) Pyk2 (Qian *et al.*, 1997) and WASp (Badour *et al.*, 2004) have been shown to be involved in cytoskeletal rearrangement. Therefore it seemed plausible that Fyn deficiency could influence IL-2 production by increasing the efficiency of stable conjugate formation, and thus the duration of signal delivered to the T cell.

To this end conjugate formation by F5 CD8 T cells was measured by a FACS based method. This involved labelling peptide pulsed APC with an intracellular fluorescent

dye and T cells with a dye, fluorescing in a distinct channel. These populations were then mixed at a 1:1 ratio and centrifuged to form conjugates. The stability of the conjugates was assessed by vigorously resuspending the cell pellet and incubating cells for 10 min at 37°C / 5% CO_2. Conjugation of F5 T cells with APC but no peptide did not lead to any double positive cell conjugates (Figure 4.5A, left panel). When F5 cells were centrifuged with peptide pulsed APC, we were able to detect doublet cells in the top right quadrant (Figure 4.5A, right panel). From Figure 4.5B it is evident that the frequency of F5 T cells forming conjugates with peptide pulsed APC increased with rising antigen concentration, however the dose response was not altered in the absence of Fyn. These data suggested that any elevation in IL-2 production by F5 $Fyn^{-/-}$ cells was unlikely to be a result of increased signal duration due to improved formation of T cell - APC stable conjugates.

4.6 Ca^{2+} signals are normal in response to antigen in the absence of Fyn

Having established that the increase in IL-2 production in the absence of Fyn was most likely due to changes in how a given signal was interpreted rather than gross changes in the interaction with antigen, we wanted to look at the pathways regulating IL-2 production. In terms of the early factors, the IL-2 promoter contains two NF-AT binding sites (Nolan, 1994) and mutations in both, leading to loss of binding is needed to abrogate promoter function (Zhang and Nabel, 1994; Thompson *et al.*, 1992). Also use of a dominant negative NF-AT molecules is able to reduce IL-2 production (Chow *et al.*, 1997). NF-AT activity is controlled via the TCR by the

cleavage of PIP_2 by the enzyme PLC-γ1 generating DAG and IP_3 (Nishibe *et al.*, 1990). IP_3 induces the mobilisation of intra-cellular Ca^{2+} that activates calcineurin, which in turn dephosphorylates NF-AT allowing it to enter the nucleus and bind to the promoter of the *Il2* gene (Aramburu *et al.*, 2000; Whitney and Sutherland, 1972). IL-2 production by T cells is also reliant on sustained Ca^{2+} mobilisation (Wacholtz and Lipsky, 1993; Goldsmith and Weiss, 1988), presumably to regulate NF-AT activity (Timmerman *et al.*, 1996). Both Lck and Fyn have been implicated as activators of PLC-γ 1 (Fusaki *et al.*, 1994; Liao *et al.*, 1993; Weber *et al.*, 1992) and thus disregulation of these molecules could affect NFAT activation through Ca^{2+} dependent pathways.

It was possible that the strength and/or duration of antigen induced Ca^{2+} mobilisation was increased in F5 $Fyn^{-/-}$ T cells. This could explain why they were able to produce more IL-2 compared to F5 WT cells after identical periods of antigenic contact (Figure 4.3C). The release of intracellular Ca^{2+} can be readily measured at the single cell level using flow cytometric techniques. To this end F5 WT and $Fyn^{-/-}$ CD8 cells were loaded with the Ca^{2+} indicator dye Indo-1 and a flux was induced by conjugating T cells with NP68 pulsed APC. From Figure 4.6A and 4.6B it is evident that conjugation with pulsed APC was able to induce a comparable Ca^{2+} flux in both groups in terms of intensity and duration. This was peptide specific, as conjugation with unpulsed APC induced no flux in either group.

To further confirm that Ca^{2+} signalling was not altered in the absence of Fyn, we measured the transcription of NF-ATc1. NF-ATc2 is expressed in resting T cells, however NF-ATc1 expression is induced after stimulation (Wang *et al.*, 1995; Northrop *et al.*, 1994). The transcription of NF-ATc1 is regulated by activation of NF-ATc2 (Zhou *et al.*, 2002), itself a direct downstream target of Ca^{2+} signalling, therefore expression of NF-ATc1 was assessed using real-time PCR. From Figure 4.6C it is evident that the detection of steady state NF-ATc1 mRNA reached maximal levels by 180 min in both groups, then dropped by 240 min. From the experiment shown in Figure 4.6C, there was a slight increase in NF-ATc1 mRNA levels in the absence of Fyn at 1 hour compare to WT, however this was not a reproducible difference. In concordance with the Ca^{2+} flux data in response to pMHC, Fyn deficiency did not seem to exert an effect on the proximal or distal aspects of Ca^{2+} signalling.

The ability of $Fyn^{-/-}$ cells to flux calcium in response to anti-CD3 was reported to be reduced in the absence of Fyn (Appleby *et al.*, 1992; Stein *et al.*, 1992). As shown in chapter 3, the use of anti-CD3 antibodies as a stimulus led to a hypo-responsive phenotype, however if CD8 was co-ligated with the TCR then cell triggering was restored and IL-2 production was elevated in the absence of Fyn. Therefore we asked whether co-ligating CD8 with CD3 would overcome the defect in Ca^{2+} mobilisation reported in $Fyn^{-/-}$ cells stimulated with anti-CD3 alone. To this end F5 WT and $Fyn^{-/-}$ were loaded with Indo-1 and pre-labelled with biotinylated antibodies against anti-CD3, anti-CD8 or anti-CD3 and anti-CD8 together. A Ca^{2+} flux was induced using

avidin to cross-link the bound antibody. From Figure 4.6D it is clear that anti-CD3 alone was able to induce a small Ca^{2+} flux in F5 WT cells, however cross-linking CD3 and CD8 was able to improve the speed of initiating the flux and also the level. Figure 4.6E shows that, as reported previously for polyclonal cells, F5 $Fyn^{-/-}$ cells mounted a slower and reduced flux in response to anti-CD3 alone compared to F5 WT cells with the same stimulus. However, cross-linking CD8 and CD3 was able to improve the response of F5 $Fyn^{-/-}$ cells generating a comparable flux to F5 WT cells with the same stimuli. These data again underscore the importance of CD8 in restoring any signalling defects observed in $Fyn^{-/-}$ cells after TCR stimulation.

4.7 Erk phosphorylation is sustained in antigen activated F5 $Fyn^{-/-}$ cells

Due to the fact that elevated IL-2 production in the absence of Fyn could not be attributed to changes in Ca^{2+} mobilisation we wanted to look at other pathways controlling factors responsible for regulating IL-2 production downstream of the TCR and CD8. It has been proposed that sustained Erk activation is important for T cell activation and effector differentiation (Berg *et al.*, 1998). Downstream of Erk are members of the Ets family of transcription factors that regulate transcription of the AP-1 family members (Rincon and Flavell, 1994). Specifically, the Erk target Elk-1 has been shown to be important to the transcription of the *c-Fos* gene (Marshall, 1995; Marais *et al.*, 1993; Gille *et al.*, 1992), thus Erk may influence the ability to produce more IL-2. Activation of Erk is controlled by Mek, which is in turn downstream of Grb2 / SOS and RasGRP activation (Aronheim *et al.*, 1994). As Lck

and Fyn are upstream of these molecules it was possible that Erk activity could be altered in the absence of Fyn.

To determine the influence of Fyn deficiency on Erk activation, cells were stimulated on a layer of peptide-loaded dimers for defined time points. At each point, prior to cell lysis, a sample of cells was stained for the expression of the activation markers CD69 and CD25 to check the level of stimulation between samples and groups. From Figure 4.7A it is clear that the cells activated comparably and, from Figure 4.7B, that the induction of phospho-Erk1 and 2 could be detected in the lysates of these samples. However, when the degree of pErk1 (Figure 4.7C) and pErk2 (Figure 4.7E) was quantified using densitometry it appeared that in the absence of Fyn, Erk activation was prolonged compared to WT controls. This late period of Erk activation between 2 and 8 hrs correlated with the period of IL-2 production by activated F5 cells (Figure 4.2).

To compare the differences in phospho-Erk induction in three independent experiments, values obtained for WT cells were set at 100% for every time point and the values for the $Fyn^{-/-}$ signal was expressed as a percentage of WT values. This corrected for variation in the WT values over the three independent experiments. Figures 4.7D and 4.7F show that less phospho-Erk1 and 2 signal was detected after activation in the absence of Fyn at 5 and 15 min over 3 independent experiments, however by 1 hour $Fyn^{-/-}$ values were similar to WT. At later time points, the pErk1/2 signal was greater than WT in the absence of Fyn. Collectively, these data show that

Fyn deficiency resulted in decreased phosphorylation of Erk1/2 before 1 hour of activation, but elevated phosphorylation between 2 and 9 hrs.

4.8 The kinetics of c-Rel nuclear translocation is normal in the absence of Fyn.

Although proximal Erk phosphorylation was reduced in the absence of Fyn it was interesting that distal Erk signal seemed prolonged. Work by Iwashima and colleagues has proposed that the activity of Erk ~4 hrs after TCR activation is essential to IL-2 production whereas proximal Erk activity may be dispensable. They based these conclusions on the observation that the use of an inhibitor of the upstream kinase Mek was able to abrogate IL-2 production when given to the cells after 4 hrs of stimulation, but had little or no effect if given from 0 to 4 hrs (Koike *et al.*, 2003). Treatment with the Mek inhibitor also blocked the nuclear translocation of the NF-κB transcription factor c-Rel. This molecule is important for IL-2 production because T cells from c-Rel$^{-/-}$ mice produce 50% less IL-2 upon stimulation, but show no defect in the upregulation of CD25 and CD69 (Kontgen *et al.*, 1995). It was interesting, therefore, that Fyn deficiency also specifically affected IL-2 production without altering activation marker expression.

To ask if the increase in distal Erk activation in the absence of Fyn correlated with an increased ability for c-Rel to translocate to the nucleus we decided to measure this event in F5 T cells using fluorescent confocal microscopy. Figure 4.8A shows the pattern of staining obtained using control rabbit sera incubated with the secondary

Alexa-440 conjugated Fab^2 anti-rabbit antibody (upper panels). In resting T cells and also 1 hour after stimulation c-Rel:Alexa 440 staining was excluded from the nucleus in both groups as judged by overlaying the Alexa-440 and PI signals from the images of the same cells. However, after 4 hrs of antigen activation, c-Rel was now present in the nucleus. Moreover the pattern of c-Rel signal followed the typical pattern observed for a nuclear localised transcription factor as, although clearly in the nucleus, it remained excluded from regions of heterochromatin (dark red areas). We also enumerated the cells positive for nuclear c-Rel in each group at 4 hrs and found there to be no overt difference (data not shown). However, these data did not rule out that subtle changes in the translocation of c-Rel had occurred, possibly in a quantitative rather than qualitative manner.

Although c-Rel is present in a resting T cell, it has been suggested that only *de novo* synthesised molecules can translocate to the nucleus and thus are important for IL-2 production (Kane *et al.*, 2002; Venkataraman *et al.*, 1995). To this end we used real time PCR to measure the steady state levels of c-Rel mRNA in response to antigen activation, as this may represent the most relevant pool of c-Rel within the cell. Figure 4.8B shows that the levels of c-Rel transcripts peaked in both groups at 180 min (3 hrs) after stimulation, preceding the detection of nuclear c-Rel protein at 4 hrs (Figure 4.7A). These data suggested that in the absence of Fyn c-Rel mRNA levels were normal, there was a suggestion that $Fyn^{-/-}$ cells contained more c-Rel mRNA at ~300 min, however the differences were comparatively small. Furthermore, the

transcription of c-Rel is likely to be regulated by calcium signals through NFAT (Venkataraman *et al.*, 1995), and we observed no gross defect in this pathway either.

4.9 Inhibition of distal Erk activity does not eliminate elevated IL-2 production by F5 Fyn$^{-/-}$ cells.

Analysis of c-Rel nuclear translocation did not reveal any gross differences in the level of nuclear c-Rel or timing of translocation in the absence of Fyn. However, confocal microscopy is not quantitative enough to determine whether more c-Rel translocated to the nucleus in the absence of Fyn. Therefore to determine if the sustained phospho-Erk level shown in the previous experiments were responsible for the elevated IL-2 production in the absence of Fyn we used the Mek inhibitor PD90859 to inhibit Erk activation for specific windows of time during the period of antigen activation (Koike *et al.*, 2003). We titrated the concentration of the inhibitor and found that 20 μM gave maximal inhibition of IL-2 production without affecting cell viability (data not shown). Figure 4.9A shows that treatment with 20 μM PD90859 for the entire culture period with peptide completely eliminated IL-2 production in both groups, indicating that IL-2 production was ultimately dependent on Erk signals. In contrast inhibition between 0-4 hrs (the period that western blotting revealed Erk activity to be maximal) marginally reduced the percentage of cells producing IL-2 (Figure 4.9B) but had no affect on the level of production per-cell (Figure 4.9C) in both F5 WT and Fyn$^{-/-}$ cells compared to uninhibited controls. It should also be noted that cells exposed to PD90859 for 0-4 hrs had been treated for

30 min before stimulation to try and ensure that Erk activity was inhibited prior to activation. Administration of the Mek inhibitor from 4 to 6 hrs marginally reduced the % and MFI of IL-2 production in both groups. However when the inhibitor was administered between 6 to 10 hrs post activation there was a profound reduction in both the percentage of IL-2 producing cells and the MFI in both groups compared to untreated controls. Interestingly, while the percentage of cells producing IL-2 was comparable between the PD90859 treated cells, the MFI in the absence of Fyn, although reduced, remained above that of F5 WT cells (Figure 4.9C). Collectively these data suggested that although Erk activation between 6-10 hrs post activation and beyond, was essential to IL-2 production in both groups, it was not responsible for dictating the increase in IL-2 production by F5 $Fyn^{-/-}$ cells.

4.10 Jnk phosphorylation is detectable in the absence of Fyn

Considering the previous data and the fact that the ability of $Fyn^{-/-}$ cells to produce elevated IL-2 could be set up as early as 1 hour after stimulation (see Figure 4.3C) we wanted to look at signalling molecules that influence IL-2 transcription at an early phase of T cell activation. One such molecule is Jnk, which has been shown to play a role in the transcription of IL-2 (Ip and Davis, 1998). T cells express Jnk1 and Jnk2, both of which exist in two different phospho-variants of p55 and p46 (Ip and Davis, 1998). Upstream of Jnk are MKK4, MKK7 (Tournier *et al.*, 2001; Yang *et al.*, 1997; Derijard *et al.*, 1995), which are in turn regulated by PKCθ and VAV (Moller *et al.*, 2001; Ghaffari-Tabrizi *et al.*, 1999), which are also influenced by Src kinases (Liu *et*

al., 2000) (Huang *et al.*, 2000; Michel *et al.*, 1998). Furthermore Flavell and colleagues have shown that while Jnk1$^{-/-}$ CD8 cells make less IL-2 after TCR stimulation, Jnk2 deficient CD8 T cells produce more IL-2 upon stimulation (Conze *et al.*, 2002). To this end we stimulated cells as described for Figure 4.8, and lysed them for biochemical analysis of Jnk phosphorylation to determine if there were defects in phosphorylation of either of the two Jnk isoforms. Figure 4.10A shows that inducible phosphorylation of the Jnks was achieved after 5 and 30 min in both groups. We were able to resolve the p46 form of Jnk1 and Jnk2, as well as a band at ~54kD that was most likely the p54 form of Jnk1/2. When these band intensities were quantified relative to loading controls, it was evident that the loss of Fyn had a minimal effect on induction of the Jnk1/2 p54 variant (Figure 4.10B), with the signal peaking after 30 min in both groups. There was possibly a slight increase in the duration of phosphorylation in the absence of Fyn with levels returning to that of WT by 9 hrs. In contrast, the phosphorylation of the p46 forms of Jnk2 (Figure 4.10C) and Jnk1 (Figure 4.10D) peaked at 5 min in WT cells, but were slightly more phosphorylated in the absence of Fyn, with maximal levels observed after 30 min of activation. Again there was also a possible increase in the duration of the phosphorylation of p46 Jnk2/1 in Fyn$^{-/-}$ cells compared to WT. Collectively, these data suggested that Fyn deficiency led to inducible levels of p46 Jnk1, p46Jnk2 and p54 Jnk1/2 phosphorylation. There may have been subtle differences but this would need confirming by replicate experiments.

4.11 Activated Fyn$^{-/-}$ and WT F5 cells express differential mRNA levels of AP-1 family members.

Jnk has been shown to influence IL-2 production through the activation of c-Jun, a component of the AP-1 transcription factor (Conze *et al.*, 2002; Wang *et al.*, 2000; Chen *et al.*, 1994). The expression of c-Jun is regulated, in part, by the activation of pre-existing c-Jun in the resting T cell by Jnk (Derijard *et al.*, 1994). In a resting T cell, c-Jun is bound as a hetero-dimer with ATF-2 to the c-Jun SRE (Rozek and Pfeifer, 1993; van Dam *et al.*, 1993), and when phosphorylated by Jnk the complex is active and transcribes the c-Jun gene. As there was a suggestion that phosphorylation of certain Jnk isoforms was elevated in the absence of Fyn, we asked if the levels of steady state c-Jun mRNA were also increased as this could, in theory, contribute to the elevated IL-2 levels. To this end, we measured c-Jun mRNA levels by real time PCR. In activated F5 WT cells, levels of c-Jun mRNA (Figure 4.11A) peaked after ~15 min of stimulation. In contrast, we were unable to detect comparable levels of c-Jun mRNA in activated F5 Fyn$^{-/-}$ cells and there was a ~4-fold reduction compared to WT cells at 15 min.

c-Jun is not the only component of the AP-1 complex that is transcribed *de novo* in an activated T cell. Transcription of other members of the AP-1 transcription family have also been shown to occur within one hour of T cell activation (Radler-Pohl *et al.*, 1993; Holt, 1992). These components form homo and heterodimers that can bind and contribute to the activation the *Il2* gene (Glover and Harrison, 1995; Jain *et al.*,

1992; Angel and Karin, 1991; Ryseck and Bravo, 1991)(Serfling *et al.*, 1989). To address the impact that Fyn may have on the transcription of these other AP-1 complex components we measured the kinetics of mRNA levels after activation using real time PCR. Figure 4.11B shows that maximal JunB mRNA was comparable between F5 WT and $Fyn^{-/-}$samples, however peak mRNA levels were detected in activated F5 $Fyn^{-/-}$ after 45 min compared to 30 min in F5 WT cells. When analysing c-Fos (Figure 4.11C), F5 WT cells contained ~0.25-fold higher mRNA levels compared to F5 $Fyn^{-/-}$ cells at the maximal time point, which was 30 min for WT cells and 45 min for $Fyn^{-/-}$. In contrast to c-Jun, FosB mRNA levels were elevated 3-fold in the absence of Fyn compared to WT cells. This was a pattern that we observed in two independent experiments. These data suggest that in Fyn deficient cells there may be a preferential bias toward AP-1 complexes consisting of FosB-Jun heterodimers as opposed to c-Jun homodimers, or c-Jun-c-Fos heterodimers.

4.12 Discussion

The aim of work presented in this chapter was to determine the cause of elevated IL-2 production by F5 $Fyn^{-/-}$ cells after antigen stimulation. Although, there was not a detailed biochemical analysis conducted of every pathway downstream of the TCR/CD8 capable of modulating IL-2 production, a number of observation were made that could serve as a basis for explaining why F5 $Fyn^{-/-}$ cells were able to make more IL-2.

Activation of F5 WT and Fyn$^{-/-}$ using optimal stimulation conditions showed that while we were able to induce the expression of activation markers in ~90% of all cells, only a proportion were also able to express IL-2. As shown in chapter 3, the percentage of activated F5 Fyn$^{-/-}$ cells making IL-2 was greater than F5 WT cells. IL-2 production required a duration of stimulation compared to CD69 expression. However, in the absence of Fyn, cells only required 1 hour of antigenic exposure to induce IL-2 production, whereas WT cells required at least 5 hrs. Collectively these data suggested that different signalling thresholds exist to control activation marker and IL-2 expression, and that Fyn deficiency improved the ability of F5 cells to cross the second threshold and produce IL-2. The use of the Src kinase inhibitor PP2 revealed that, although 1 hour of antigen contact was enough to induce IL-2 production by F5 Fyn$^{-/-}$ cells, they required Src kinase activity for longer than 3 hrs stimulation in order to produce IL-2. The elevated IL-2 production in the absence of Fyn was most likely not influenced by the efficiency of conjugate formation, as F5 Fyn$^{-/-}$ cells did not show an improved ability to form stable conjugates with peptide pulsed APC compared to WT. When we looked at intracellular events, the induction and duration of Ca^{2+} mobilisation was also unaffected in the absence of Fyn, however we did find that Erk activation was prolonged compared to WT cells. Downstream of Erk, confocal analysis revealed that the kinetics of c-Rel nuclear translocation were comparable in both groups. Furthermore, administration of the Mek inhibitor PD90859 during the period of prolonged Erk phosphorylation was not able to eliminate the ability of F5 Fyn$^{-/-}$ to make more IL-2 than WT cells, suggesting that this was not the underlying cause of the phenotype. Analysis of Jnk activation

revealed that phosphorylation was not defective in the absence of Fyn, however there may have been a subtle increase in the levels of p46 Jnk1/2. The expression of components of the AP-1 complex revealed that, in the absence of Fyn, there was increased levels of FosB mRNA and reduced levels of c-Jun and c-Fos mRNA compared to F5 WT cells. Collectively these data suggest that Fyn deficiency may lead to elevated IL-2 production by uncoupling the need for prolonged antigenic exposure to drive Src kinase activation and also possibly through the production of differential AP-1 family members.

In terms of the influence that Fyn deficiency had on the mRNA levels of AP-1 members, studies have shown that c-Jun can form homodimers with itself and heterodimers with members of the Fos family (Kouzarides and Ziff, 1989; Kouzarides and Ziff, 1988). The stability of Jun-Fos heterodimers is greater than Jun-Jun homodimers (Smeal *et al.*, 1989). Moreover, the binding affinity to AP-1 sites of Jun-Fos complexes is greater than Jun-Jun homodimers (Ryseck and Bravo, 1991; Ransone *et al.*, 1990b; Ransone *et al.*, 1990a; Halazonetis *et al.*, 1988). This could be highly significant to the transcriptional control of the IL-2 gene as the AP-1 sites within the promoter are relatively weak non-consensus sites (Hentsch *et al.*, 1992; Jain *et al.*, 1992; Serfling *et al.*, 1989). Therefore Jun-Fos heterodimers are able to bind with greater affinity than Jun-Jun homodimers to these sites and may be better transcriptional activators, thus the induction of Fos members may be essential to increasing IL-2 production in a T cell. There are several lines of evidence for this. Firstly, Fos members are transcribed *de novo* after T cell activation (Jain *et al.*, 1992;

Kovary and Bravo, 1991) and IL-2 gene transcription can be blocked by the use of protein synthesis inhibitors (Shaw *et al.*, 1987). Secondly, FosB has been shown to specifically improve the binding of AP-1 complexes (Zerial *et al.*, 1989). Also over-expression of plasmid-encoded FosB was able to improve IL-2 reporter gene transcription in T cell lines and also in non-T cells (Hentsch *et al.*, 1992). Furthermore, transgenic over-expression of c-Fos in mice led to increased IL-2 mRNA (Ochi *et al.*, 1994). Also the use of dominant negative c-Jun lacking the DNA binding domain was able to reduce IL-2 promoter activity, possibly by sequestering Fos sub-units away from the AP-1 sites (Ransone *et al.*, 1990b). The importance of AP-1 affinity to IL-2 induction was shown by the conversion of the non-consensus sites within the IL-2 minimal enhancer to consensus sequences that were able to bind the lower affinity Jun-Jun AP-1 complexes. This greatly improved AP-1 driven transcription of an IL-2 reporter gene (Hentsch *et al.*, 1992). Furthermore, studies have suggested that different stimuli may induce differential expression of AP-1 members changing the composition of the complex (Brooks *et al.*, 1995; Chatta *et al.*, 1994; Rincon and Flavell, 1994; Ullman *et al.*, 1993). It is also of interest that in anergic T cells that fail to make IL-2, there is a specific defect in the transcription of Fos members (Mondino *et al.*, 1996). As outlined in chapter 3, Fyn has been implicated in anergy and may thus regulate the ability of a T cell to make IL-2 by reducing expression of FosB and increasing expression of c-Jun, because in the absence of Fyn we observed the complete opposite effect. Therefore the composition of AP-1 may be the decisive factor for the duration and level of IL-2 expression by an activated T cell.

The effect that increased AP-1 binding would have on the *Il2* gene is not solely restricted to AP-1 sites. Work by E. Rothenburg's group has shown that, if NFAT activation was blocked using CsA then the binding of all factors was inhibited, as measured by DNA foot-printing (Garrity *et al.*, 1994). A similar result was obtained by blocking NF-κB activation using Forskolin (Chen and Rothenberg, 1994). Collectively, these data suggested that binding to the *Il2* promoter is an all or nothing event, whereby the loss of binding of one molecule will prevent the rest from assembling on the promoter. Furthermore, it is well established that binding of the respective factors to the distal Oct-1 site (de Grazia *et al.*, 1994) and the proximal NFAT sites are regulated by the co-operative binding of AP-1 factors (Masuda *et al.*, 1995; Yaseen *et al.*, 1994; Jain *et al.*, 1993). Moreover, there is evidence that the specific composition of AP-1 will have an effect on the binding of factors to these sites (McCaffrey *et al.*, 1993; Ullman *et al.*, 1993; Ullman *et al.*, 1991). Interestingly, the use of a dominant negative c-Jun molecule that retains the DNA binding motif, but lacks the transactivating motif was able to inhibit the binding of factors at the NFAT- AP-1 composite site but not the Oct-1-AP-1 site (Petrak *et al.*, 1994). The c-Jun molecule lacking the transactivating domain would render Jun-Jun homodimers ineffective, but not Fos-Jun dimers suggesting that the co-operative nature of AP-1 at the Oct-1-AP-1 site is regulated by the specific composition of the AP-1 dimer. These data support the view that altering the AP-1 composition and the subsequent binding affinity can also influence the binding of other *Il2* transcriptional regulators due to the co-operative nature of AP-1.

Interestingly a study by Greenberg and colleagues suggested that CD8 cells incapable of making IL-2 after antigenic stimulation had a specific block in the transactivation of the –150 non-consensus AP-1 site, downstream of the CD28RE (see Figure 4.1). They found that functional AP-1 complexes hade been formed in these cells as consensus AP-1 sites were transactivated. Furthermore, the mutation of the non-consensus -150 AP-1 site to a consensus site was able to drive IL-2 expression in all cells (Finch *et al.*, 2001). Collectively these data again suggest that the decision to make IL-2 is controlled, in part, by AP-1 factors that contain proteins such as FosB that can specifically bind to non-consensus sites, further suggesting that the formation of high affinity Fos containing complexes may be essential to IL-2 production.

The CD69 promoter also contains an AP-1 site that is essential to transcription (Castellanos *et al.*, 1997), but this did not seem to be affected in the absence of Fyn. If the composition of AP-1 to higher affinity FosB containing complexes occurs in the absence of Fyn we may have also expected an increase in CD69 expression. However the AP-1 binding site in the promoter of the CD69 gene is a consensus site (Castellanos *et al.*, 1997) and therefore may not be as sensitive to changes in AP-1 composition.

There were two possible explanations for the increase in FosB mRNA in the absence of Fyn. First, is that the transcription of the FosB gene could be upregulated through the promoter. Transcription of Fos members is controlled by members of the Ets-family of transcription factors that include SAP-1, Elk-1 and Nets (Shipley *et al.*,

1994). These have been shown to be pre-bound to the SRE in the promoter of Fos genes and when activated by phosphorylation drive transcription (Marais *et al.*, 1993; Gille *et al.*, 1992). The MAP kinases Erk, p38 and Jnk have been shown to regulate the functions of Elk-1 by phosphorylation (Cruzalegui *et al.*, 1999; Enslen *et al.*, 1998; Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Whitmarsh *et al.*, 1995; Janknecht *et al.*, 1994). In contrast, while p38 and Erk can phosphorylate SAP-1 (Janknecht and Hunter, 1997a; Whitmarsh *et al.*, 1997; Price *et al.*, 1996; Whitmarsh *et al.*, 1995), it is thought to be a relatively poor substrate for the Jnks (Whitmarsh *et al.*, 1997; Whitmarsh *et al.*, 1995). Jnk1 has been shown to be able to phosphorylate SAP-1, but only when its activity was increased by over-expression (Janknecht and Hunter, 1997b). However Erk is considered to be the major MAPK responsible for driving Fos expression (Marshall, 1995). In the absence of Fyn, the pathway responsible for the increase in FosB mRNA is unclear. Transcription of FosB is a relatively early event (≤45 min), therefore Erk activation may not be responsible for this process as it was actually reduced in the absence of Fyn at this time point compared to WT.

In contrast to Erk, at 30 min of stimulation, it was possible that the phosphorylation of p46 Jnk1 and Jnk2 was increased compared to WT. Increased Jnk activity could lead to elevated Elk-1 activity and increase the transcriptional regulation of the FosB gene. However, activation of Elk1 has also been shown to regulate transcription of c-Fos (Cavigelli *et al.*, 1995), and as the steady state of c-Fos mRNA was actually decreased in the absence of Fyn, it would be unlikely that Elk-1 activity was responsible. It is possible that Jnk may be influencing other Ets family members as

when Jnk1 is over-expressed, it can activate SAP-1 (Janknecht and Hunter, 1997b). SAP-1 is also capable of controlling the expression of Fos family members (Janknecht and Hunter, 1997a), however the molecule(s) responsible for driving the increase in FosB expression would have to be distinct from those regulating c-Fos expression due to the different patterns we observed in the mRNA levels of these genes.

A second possible explanation for the increase in FosB mRNA in the absence of Fyn is through changes in the stability of the transcripts. Fos transcripts contain AU-rich elements (ARE) in the 3' region of the gene (Wilson and Treisman, 1988). These make the transcripts unstable and prone to degradation (Wilson and Treisman, 1988). It is possible to classify transcripts containing ARE into three classes on the basis of the AUUUA repeat patterns (Chen and Shyu, 1995; Chen *et al.*, 1995; Chen and Shyu, 1994; Stoecklin *et al.*, 1994). There is also evidence that the mechanisms controlling the stability of these classes of transcripts may differ as mice lacking a protein known to interact with these ARE led to the selective increase in TNFα and GMCSF mRNA (Carballo *et al.*, 2000; Taylor *et al.*, 1996). Fos members are members of class I ARE (Chen *et al.*, 1995) and thus the mechanisms regulating mRNA degradation are most likely shared between all the Fos members. Therefore, if it were simply a case of increased stability of the mRNA rather than increased transcription of the gene that was responsible for the elevated FosB levels in the absence of Fyn, it was interesting that c-Fos mRNA was actually reduced compare to WT. Our data also suggested that although FosB levels were increased in the absence

of Fyn, the mRNA levels then rapidly decreased by 60 min compared to WT cells. A possible explanation for this result is that FosB has been shown to silence its own promoter (Lazo *et al.*, 1992).

If increased Jnk activity in the absence of Fyn was responsible for the increase in FosB gene transcription, this would mean that there would have to be a pathway for regulating Fos levels that would be ultimately dependent on Src kinase activity. A study by Mustelin and colleagues highlighted a potential relationship between the regulation of Src kinase activity and transcription of Fos family members by Jnk proteins (Gjorloff-Wingren *et al.*, 1999). They found that transfection of Jurkat cells with the phosphatase PEP, which regulates Lck/Fyn activity, led to a decrease in the phosphorylation of Tyr394 in Lck, a reduction in the activation and function of Jnk, reduced expression of a reporter gene linked to the Fos promoter and finally reduced IL-2 production (Gjorloff-Wingren *et al.*, 1999). In relation to this, the use of a Csk siRNA was able to increase Lck activity and IL-2 production by spontaneous NF-AT/AP-1 activation (Vang *et al.*, 2004). Furthermore, there is also evidence of Src kinase dependent pathways that lead to the preferential transcription and activation of FosB in B cells downstream of the BCR (Dickinson *et al.*, 1995).

As well as increased FosB expression, we observed a reduction in c-Jun expression. Analysis of c-Jun$^{-/-}$ T cells using the Rag complementation system revealed that, although IL-2 production was partially reduced, c-Jun was not essential to production of this cytokine (Chen *et al.*, 1994). Similar to the increase in FosB mRNA, the

decrease in c-Jun mRNA may be due to changes in the transcription of the gene, or due to alterations in the mRNA stability. In terms of the latter, it is possible that we could observe a selective decrease in c-Jun mRNA stability while observing increase in Fos member mRNA stability. Studies have suggested that Jun mRNA are class III ARE where as Fos mRNAs are class I ARE on the basis of the AUUUA patterns within these regions (Chen and Shyu, 1994) and therefore may be subject to different signals for stability. However, the same argument used for the Fos proteins applies to the Jun proteins, namely that c-Jun and JunB are in the same class of ARE, therefore it seems strange that we observed decreased c-Jun, but relatively normal JunB compared to WT cells in the absence of Fyn if the mechanisms controlling stability are shared.

With respect to transcriptional control of c-Jun, in resting cells, pre-existing c-Jun protein is found bound to the promoter of the *c-Jun* gene, along with the transcription factor ATF-2 (Rozek and Pfeifer, 1993). Phosphorylation of Ser-63 and Ser-73 within the N-terminal activation domain of c-Jun by Jnk, and phosphorylation of ATF-2 by Jnk and p38 allows this transcription factor complex to become activated and drives transcription (van Dam *et al.*, 1995; Wilhelm *et al.*, 1995; Minden *et al.*, 1994). It was possible that we observed a degree of elevated Jnk activation so it was conflicting that this correlated with a decrease in c-Jun mRNA. There are a number of other possible explanations for the decrease in c-Jun mRNA levels that may not involve Jnk activation. For example ATF-2 activation is also important to drive c-Jun expression, although this is mediated in part by Jnks may be also controlled by p38

MAPK (Derijard *et al.*, 1995). We did not address the activation of p38 in the absence of Fyn, and defects in the activation of this molecule could translate to a decrease in c-Jun transcription.

MAPK kinases not only play a role in the transcription of the components of AP-1 complexes, they are also able to control their transcriptional activity. For example Jnk can phosphorylate c-Jun and increase the transactivation potential of Jun-Jun homodimers and Jun-Fos heterodimers (Whitmarsh and Davis, 1996). Furthermore, Erk has been shown to phosphorylate a site near the basic region of c-Jun (Minden *et al.*, 1994; Chou *et al.*, 1992) that can inhibit the action of Jun-Jun homodimers, but not Jun-Fos heterodimers (Lin *et al.*, 1992; Boyle *et al.*, 1991). In the absence of Fyn, we do not observe a complete loss of c-Jun mRNA. The c-Jun present is clearly functional as we have normal CD69 expression, and because Fos members cannot form functional homodimers (Smeal *et al.*, 1989), we must assume that without functional c-Jun to form heterodimers with we would not observe any IL-2 production. As we see some upregulation of c-Jun message, albeit reduced compared to WT, we cannot rule out that there are still some Jun-Jun homodimers formed in the absence of Fyn. However the prolonged Erk activity we observed may selectively impair the function of these complexes as they may be phosphorylated in the inhibitory regions. This would then influence the activity of the AP-1 dimers formed as the prolonged Erk activity would inhibit Jun-Jun homodimers, but not Fos-Jun heterodimers.

It is also a possibility, that in the absence of Fyn, we primarily have AP-1 complexes containing JunB and FosB, as opposed to c-Jun and c-Fos as the latter both show a reduction in mRNA levels after activation. However, the function of JunB is unclear, and may even repress AP-1 transactivation when over expressed (Chiu *et al.*, 1989). Furthermore, transfected JunB failed to increase the transcription of a reporter gene when expressed in EL-4 cells (Hentsch *et al.*, 1992). More specifically, JunB has been shown to be able to down regulate expression of the *c-Jun* gene (Chiu *et al.*, 1989) and this may also contribute to the reduced levels of c-Jun in the absence of Fyn.

The idea that Erk, Jnk and possibly p38 mediated signals were controlling IL-2 production in activated CD8 cells is an attractive idea in that CD8 cells that have undergone AINR (see Chapter 3) and no longer make IL-2 show defects in Erk, Jnk and p38 activation (Tham and Mescher, 2001). However, we will need to look in more detail at MAPK activity and activation using western blotting and also *in vitro* kinase assays to assess the function of these molecules in the absence of Fyn, and how this could influence the transcription and phosphorylation of AP-1 complexes. Also in terms of Jnk1 and Jnk2 activity, it may be useful to look at the phosphorylation status of the downstream targets c-Jun and ATF-2. Studies in fibroblasts (Liu *et al.*, 2004) and T cells (Conze *et al.*, 2002) have suggested that the phosphorylation of c-Jun may be positively regulated by Jnk1 but negatively regulated by Jnk2.

In addition to the early signals that may be responsible for the changes in the mRNA of AP-1 members, Fyn may have also affected the function of late factors important to IL-2 transcription. This conclusion is based on two observations. Firstly, from the duration of stimulation experiment (Figure 4.3), 1 hour of antigen exposure was sufficient to induce IL-2 production in F5 $Fyn^{-/-}$ cell but not in WT cells. Within this time frame, the putative high affinity AP-1 complexes would have been transcribed in the absence of Fyn but transcription and activation of distal factors, should not have been induced by such a short period of antigenic contact (Venkataraman *et al.*, 1995; Northrop *et al.*, 1994; Kang *et al.*, 1992), and therefore IL-2 production should not have been detected. Indeed, F5 WT cells required at least 4 hrs of antigenic contact in order to make IL-2. Collectively, these data suggested that in the absence of Fyn, either the cells had received signals that led to the premature activation of the late factors needed for IL-2 production in the 1 hr period, or that the putative changes in AP-1 composition overcame any requirement for the activation of late factors in IL-2 transcription. Indeed c-Rel nuclear localisation could not be detected after 1 hour in the absence of Fyn, so it was unlikely that premature activation of this pathway had occurred. Furthermore, treatment of F5 $Fyn^{-/-}$ cells with the Src inhibitor PP2 after 3 hrs of activation suggested that the signals generated after 1 hour were not enough to induce IL-2 production 24 hrs later, as we observed no cytokine by intracellular staining. If the signals delivered through Src kinases to the F5 $Fyn^{-/-}$ cells after just 1 hour of activation had already dictated that they would be able to make IL-2 24 hrs later, then the administration of PP2 after 3 hrs should not have been able to prevent F5 $Fyn^{-/-}$ cells from making this cytokine. Collectively these data show that although

the F5 $Fyn^{-/-}$ cells did not require continued antigenic exposure in order to achieve IL-2 production, they did require Src kinase activity for over 3 hrs, presumably to drive the signals responsible for c-Rel / Oct2 activation and translocation. Therefore after a relatively short period of stimulation, Fyn deficient cells may continue to signal through Src kinases and no longer required TCR/CD8 ligation to drive this. This would provide an explanation as to why after stimulation, a greater frequency of F5 $Fyn^{-/-}$ cells were able to produce IL-2 as even relatively short periods of antigenic exposure would induce IL-2 production in the absence of Fyn.

It is well established that in the context of pMHC stimulations, T cell-APC interactions are extremely dynamic. So within a culture well the time a T cell spends in contact with the APC may influence the quality of signal delivered, regulating the ability to produce IL-2 (Hurez *et al.*, 2003). As mentioned previously, several Fyn specific T cell substrates have been shown to link into pathways involved in rearrangement of the cytoskeleton, an important event controlling synapse and conjugate formation. Vav has been shown to be important in cytoskeletal rearrangement, furthermore it has suggested that Fyn is responsible for phosphorylating Vav (Michel *et al.*, 1998), and that $Fyn^{-/-}$ cells are defective in their ability to form conjugates (Huang *et al.*, 2000). Although we observed no gross difference when we addressed the stability of conjugate formation, we did not look at the duration of APC - T cell contact. If it is the case that Fyn deficiency reduces the duration of conjugate formation, then shorter periods of APC contact with $Fyn^{-/-}$ T cells may not lead to activation defects, as our observations show that shorter periods

of antigenic contact would still be sufficient to induce IL-2 production in the absence of Fyn.

Another Fyn substrate that has been shown to play a role in conjugate formation is ADAP (Wang *et al.*, 2004). Expression of ADAP in a T cell line was able to greatly improve conjugate formation and LFA-1 clustering, however a mutant ADAP molecule that was unable to bind SLP-76 had the opposite effect. Moreover, not only was pSMAC formation and LFA-1 clustering affected by the dominant negative ADAP molecule, but also IL-2 production was reduced 2-fold. Interestingly, the interaction of SLP-76 with ADAP is thought to be mediated by the phosphorylation of ADAP by Fyn (Raab *et al.*, 1999), therefore it was interesting that in our hands Fyn deficiency was able to upregulate IL-2 production. It would be interesting to look at the phosphorylation status of ADAP and the interaction with SLP-76 in our cells, and also to look at the duration of conjugate formation using microscopic techniques to determine if defects in these events could influence subsequent IL-2 production.

From the data presented in this chapter, we propose the following model to explain the role of Fyn in CD8 T cell activation. Optimal stimulation of F5 WT cells via the TCR and CD8 would drive efficient signals capable of up regulating activation marker expression in 90% of cells, presumably through Lck. However Fyn would then serve to downregulate these signals, possibly through the action of the PAG/PEP/CSK complex. This would mean that only a subset of F5 WT cells would

receive enough signal to cross the second threshold and be able to produce IL-2. However, in the absence of Fyn, while a 1 hour period of stimulation may induce differential expression of high affinity AP-1 complexes this is not sufficient to induce IL-2 expression without further Src kinase activity as the cells still remained sensitive to PP2 3 hrs later. Instead without Fyn, the activity of Src kinases may continue even after the removal of antigen, presumably leading to activation of distal IL-2 factors. This may be due to an inability or reduction in the efficiency to reform the PAG/CSK/PEP complex and downmodulate Src kinase activity (see Figure 4.12).

In terms of future work, we hope to confirm that the differences in Fos-Jun mRNA levels in the absence of Fyn translate into changes in the AP-1 complex at the protein level. We plan to do this with EMSA using both consensus and non-consensus AP-1 sequences as bait, and confirm the identity of the complex using antibodies against specific Fos-Jun protein to look for super-shifts. We may expect to see the biggest differences in the composition of factors binding to the non-consensus sites in the absence of Fyn. Furthermore, we plan to transfect primary F5 T cells with or without Fyn with reporter constructs driven by either consensus or non-consensus AP-1 sites, and assay for changes in activity. This may provide an idea of the functional outcome of altering AP-1 composition.

Furthermore, to begin addressing if, after the removal of antigen, $Fyn^{-/-}$ cells continue to signal through Lck we plan to stimulate cells for 1 hour, remove them from the antigen and look at the phosphorylation status of Lck substrates such as Zap-70 and

Shc. In WT cells, we may expect to observe a decline in the phosphorylation of these molecules after removal of stimulation, however signal may persist over time in the absence of Fyn. To then provide a functional readout it may also be of interest to activate cells for 1 hour, remove again from the stimulus, and then look for c-Rel translocation into the nucleus 3 hrs later. We would expect that WT cells may contain no nuclear c-Rel after just 1 hour of antigen exposure, however if signalling continued in the absence of Fyn, then we may detect nuclear c-Rel. In terms of the PAG/CSK/PEP complex we also plan to look at the phosphorylation of Tyr317 of PAG using an antibody against this residue supplied by Dr J Lindquist (University of Magdeberg, Germany). We may expect to see that in the absence of Fyn, the rephosphorylation of this residue does occur, but with slower kinetics. We have conducted some preliminary experiments looking at the phosphorylation of the Tyr 317 residue and found that we could detect it in WT cells 6-9 hrs after activation, but no band was present in the absence of Fyn (data not shown).

Although not addressed in this chapter, there are other molecules that may exert positive influence such as Sp1-like (Skerka *et al.*, 1995; Chen and Rothenberg, 1993) or negative influence such as Nil-2A (Williams *et al.*, 1991) on the IL-2 minimal enhancer. It may also be of interest to look at the function of these molecules in future experiments. Furthermore, studies have suggested that regions of DNA upstream of the *Il2* minimal enhancer, while not able to drive transcription of linked reporter genes alone, may have modulatory effects on IL-2 production (Novak *et al.*, 1990; Serfling *et al.*, 1989; Durand *et al.*, 1988). To address if regions other than the

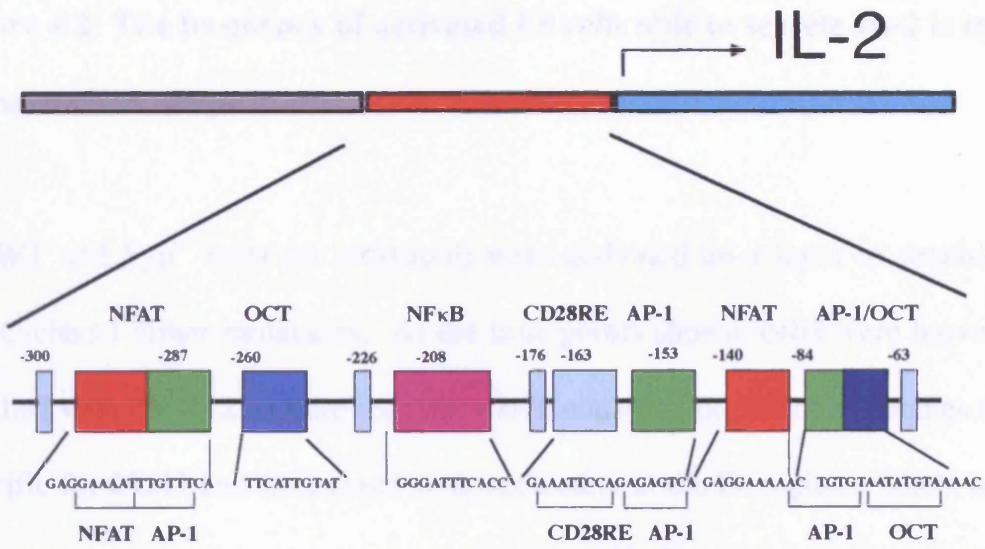
Il2 minimal enhancer are responsible for the increase in IL-2 in the absence of Fyn, we plan to use a transgenic mouse line expressing the luciferase gene under the control of the *Il2* minimal enhancer. We are in the process of crossing these to the F5 Rag-1$^{-/-}$ WT or Fyn$^{-/-}$.background. If the *Il2* minimal enhancer is enough to drive increased IL-2 transcription in the absence of Fyn, we should expect to observe increased luciferase activity after activation of the F5 Fyn$^{-/-}$ cells with peptide.

It is also a possibility that without Fyn, there is some form of alteration at the chromatin level in terms of the accessibility of the IL-2 locus. Certainly the ability of Fyn$^{-/-}$ cells to still produce more IL-2 than WT after recall PdbU and ionomycin stimulation suggested that whatever difference set up by the initial TCR/CD8 stimulus was transmitted to daughter cells, possibly at the level of chromatin modelling and locus accessibility. PdbU and ionomycin bypass TCR signalling but without restimulation, IL-2 production could not be detected, we can assume therefore that we are looking at the accessibility of the *Il2* locus, telling us what the cells have the ability to produce. Therefore it is interesting that even though IL-2 production after TCR/CD8 stimulation has ceased before cell division, the resulting Fyn$^{-/-}$ daughter cells retained the ability to make more IL-2. Studies have shown that IL-2 is monoallelically expressed (Hollander *et al.*, 1998) and therefore it could not be excluded that IL-2 production in the absence of Fyn may reflect changes in this phenomenon. It may be of interest to look at the accessibility of the IL-2 locus in Fyn$^{-/-}$ T cells.

Figure 4.1: The *Il2* minimal enhancer contains binding sites for factors that regulate IL-2 production at different stages of T cell activation

The *Il2* minimal enhancer (A) consists of a region of ~300bp upstream of the *Il2* coding sequence that can confer inducible, tissue specific expression of linked reporter constructs. The regions that bind the various factors that can regulate IL-2 production are shown. These factors have been implicated in controlling IL-2 production at proximal and distal stages of T cell activation. The model (B) is based on the observation that activation of these factors occurs either early or late in T cell activation. While early factors can drive the expression of activation markers, IL-2 production requires a more prolonged period of stimulation that may reflect the need for the late factors to be transcribed and become activated. A is adapted from Jain *et al.* (1995).

A


IL-2
NFAT
OCT
NFκB
CD28RE
AP-1
NFAT
AP-1/OCT
-300
-287
-260
-226
-208
-176
-163
-153
-140
-84
-63
GAGGAAAATTTGTTTCA
TTCATTGTAT
GGGATTTCACC
GAAATTCCAGAGAGTCA
GAGGAAAAAC
TGTGTAATATGTAAAAC
NFAT
AP-1
CD28RE
AP-1
AP-1
OCT

B

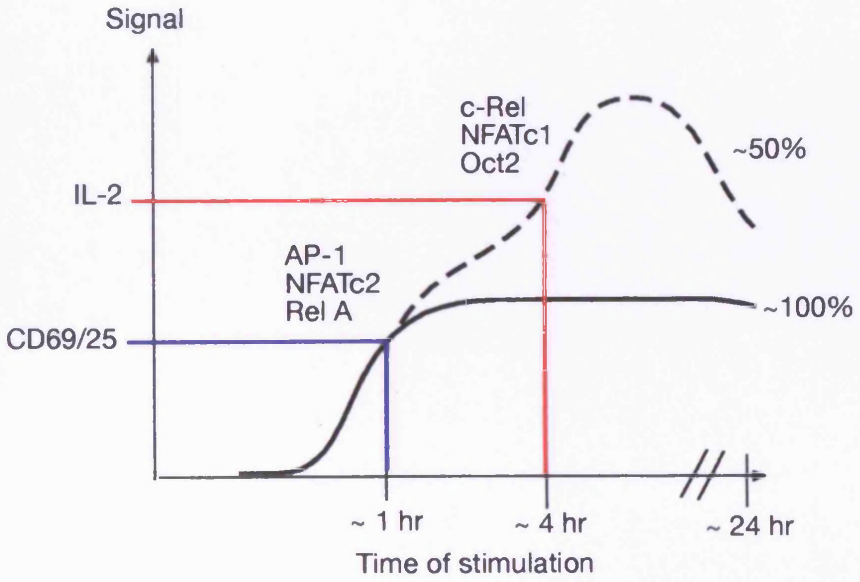

Signal
c-Rel
NFATc1
Oct2
~50%
IL-2
AP-1
NFATc2
Rel A
~100%
CD69/25
~ 1 hr
~ 4 hr
~ 24 hr
Time of stimulation

Figure 4.2: The frequency of activated F5 cells able to secrete IL-2 is increased in the absence of Fyn

F5 WT and $Fyn^{-/-}$ cells (as indicated) were activated on a layer of peptide pulsed MHC-class I dimer molecules. At the time points shown, cells were harvested and labelled with the IL-2 capture reagent, which consisted of pair of antibodies that were specific for CD45 and IL-2 fused with each other at the Fc regions. Cells were then returned to culture for a further hr and captured IL-2 protein was visualised with an anti-IL-2 PE conjugated antibody. Cells were also stained with anti-CD25 and anti-CD69 and analysed by FACS.

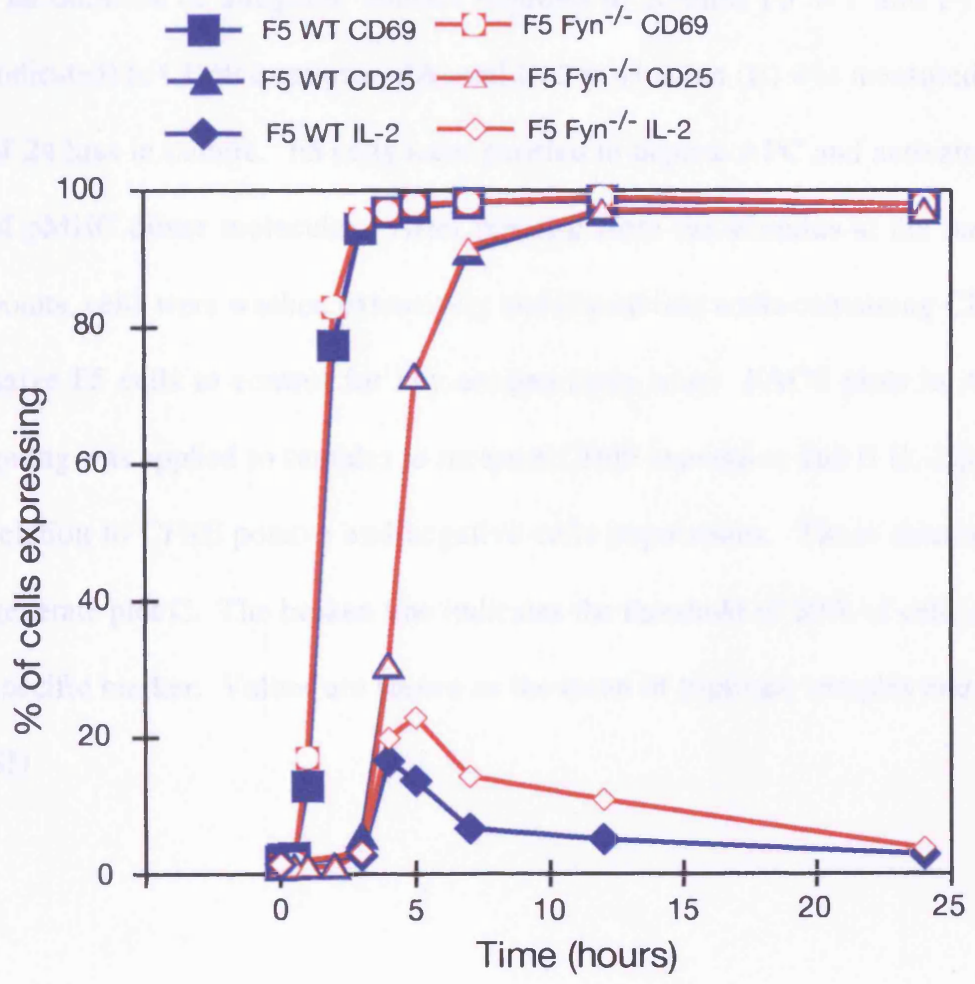
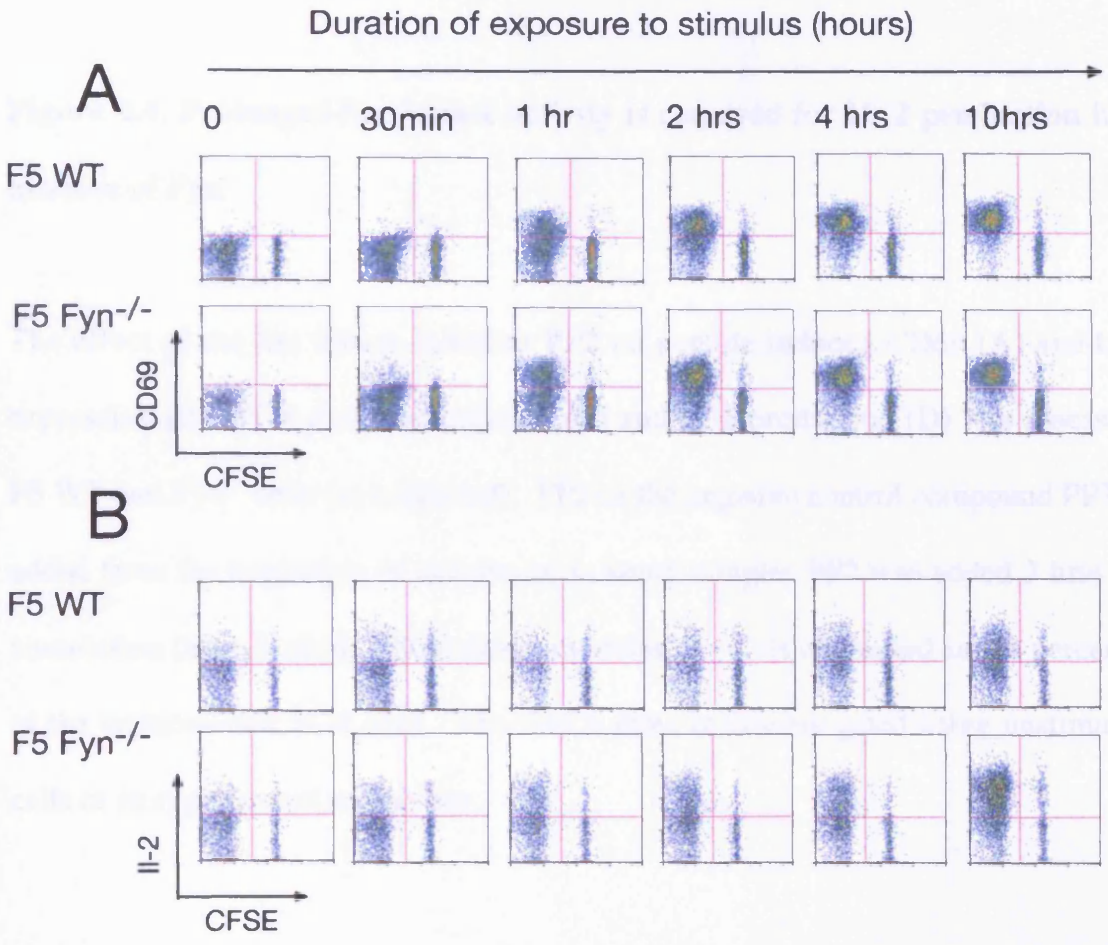

F5 WT CD69
F5 Fyn-/- CD69
F5 WT CD25
F5 Fyn-/- CD25
F5 WT IL-2
F5 Fyn-/- IL-2
% of cells expressing
100
80
60
40
20
0
0
5
10
15
20
25
Time (hours)

Figure 4.3: F5 Fyn$^{-/-}$ cells require shorter periods of antigen exposure to commit to IL-2 production

The duration of antigenic contact required to commit F5 WT and Fyn$^{-/-}$ cells (as indicated) to CD69 expression (A) and IL-2 production (B) was measured after a total of 24 hrss in culture. F5 cells were purified to deplete APC and activated on a layer of pMHC dimer molecules. After removal from the stimulus at the indicated time points, cells were washed extensively and placed into wells containing CFSE labelled naïve F5 cells to control for any antigen carry over. FACS plots in A show how gating was applied to samples to measure CD69 expression and B IL-2 production in relation to CFSE positve and negative cells populations. These data were used to generate plot C. The broken line indicates the threshold of 50% of cells expressing a specific marker. Values are shown as the mean of triplicate samples and error bars ± SD.


Duration of exposure to stimulus (hours)
A
0
30min
1 hr
2 hrs
4 hrs
10hrs
F5 WT
F5 Fyn-/-
CD69
CFSE
B
F5 WT
F5 Fyn-/-
Il-2
CFSE

C

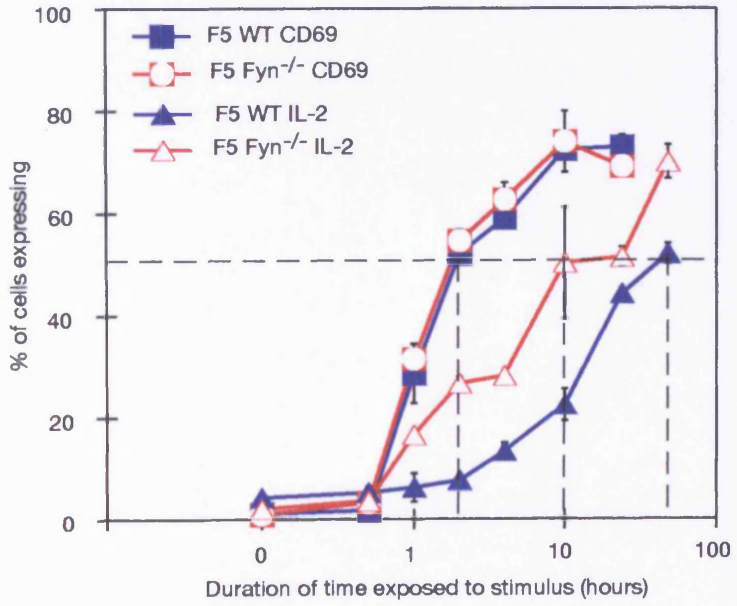

F5 WT CD69
F5 Fyn-/- CD69
F5 WT IL-2
F5 Fyn-/- IL-2
% of cells expressing
100
80
60
40
20
0
0
1
10
100
Duration of time exposed to stimulus (hours)

Figure 4.4: Prolonged Src kinase activity is required for IL-2 production in the absence of Fyn

The effect of the Src kinase inhibitor PP2 on peptide induced CD69 (A) and CD25 expression (B), TCR down-modulation (C) and IL-2 production (D) was assessed in F5 WT and $Fyn^{-/-}$ cells (as indicated). PP2 or the negative control compound PP3 was added from the beginning of culture, or in some samples PP2 was added 3 hrss after stimulation (as indicated). TCR down-modulation (C) is expressed as the percentage of the unstimulated TCR MFI. The FACS plots in D were gated using unstimulated cells or isotype control antibodies.

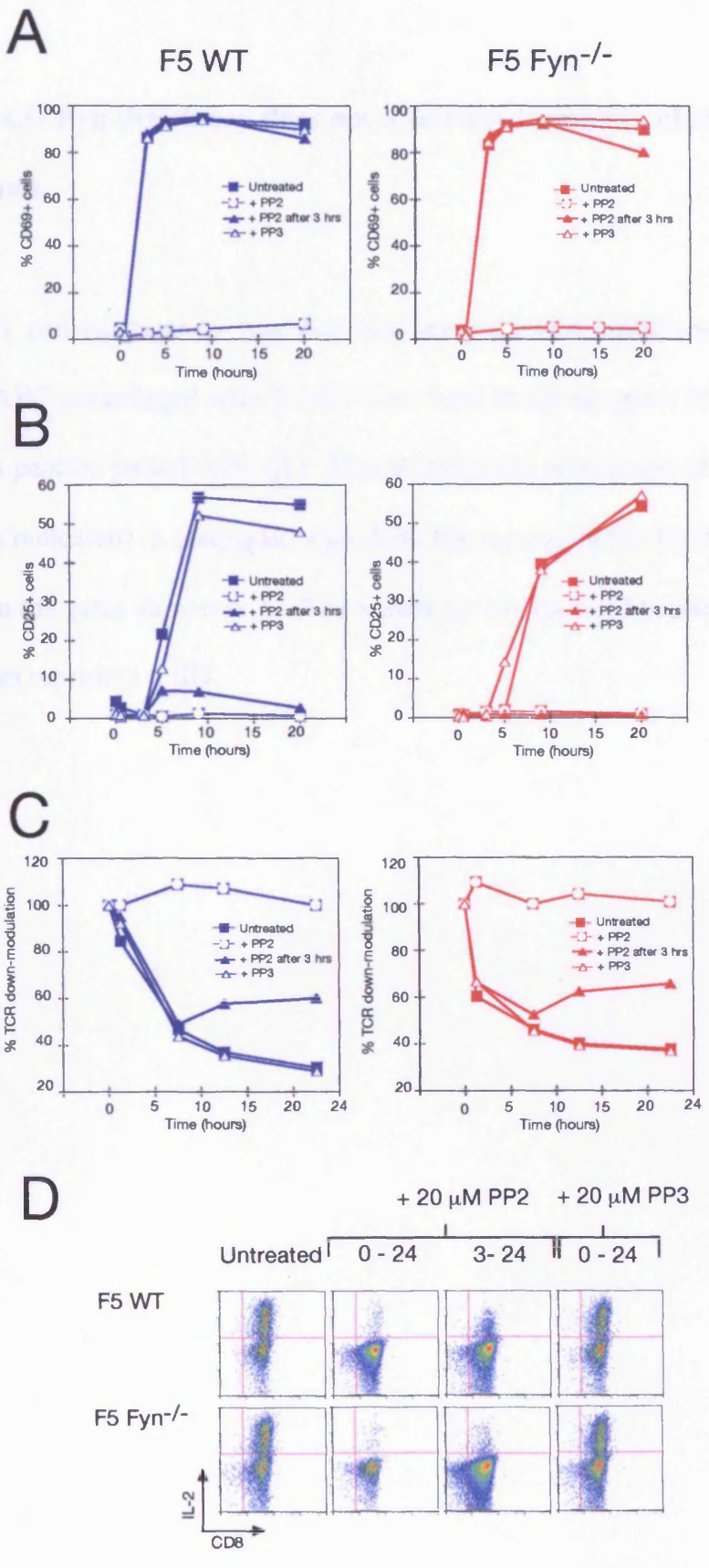

A
F5 WT
F5 Fyn-/-
% CD69+ cells
Time (hours)
Untreated
+ PP2
+ PP2 after 3 hrs
+ PP3
B
% CD25+ cells
C
% TCR down-modulation
D
+ 20 μM PP2
+ 20 μM PP3
Untreated
0 - 24
3- 24
0 - 24
IL-2
CD8

Figure 4.5: Fyn deficiency does not affect the formation of stable T cell – APC conjugates

APC – T cell conjugation was assessed using a FACS based method. Non-peptide pulsed APC centrifuged with T cells were used to set the gates to identify conjugates between peptide pulsed APC (A). Plot B shows the percentage of F5 WT and $Fyn^{-/-}$ T cells (as indicated) in conjugate with APC fluorescing in the FL-1 and FL-2 channels based on the gates shown in A. The values are the mean of triplicate samples and the error bars represent ± SD.

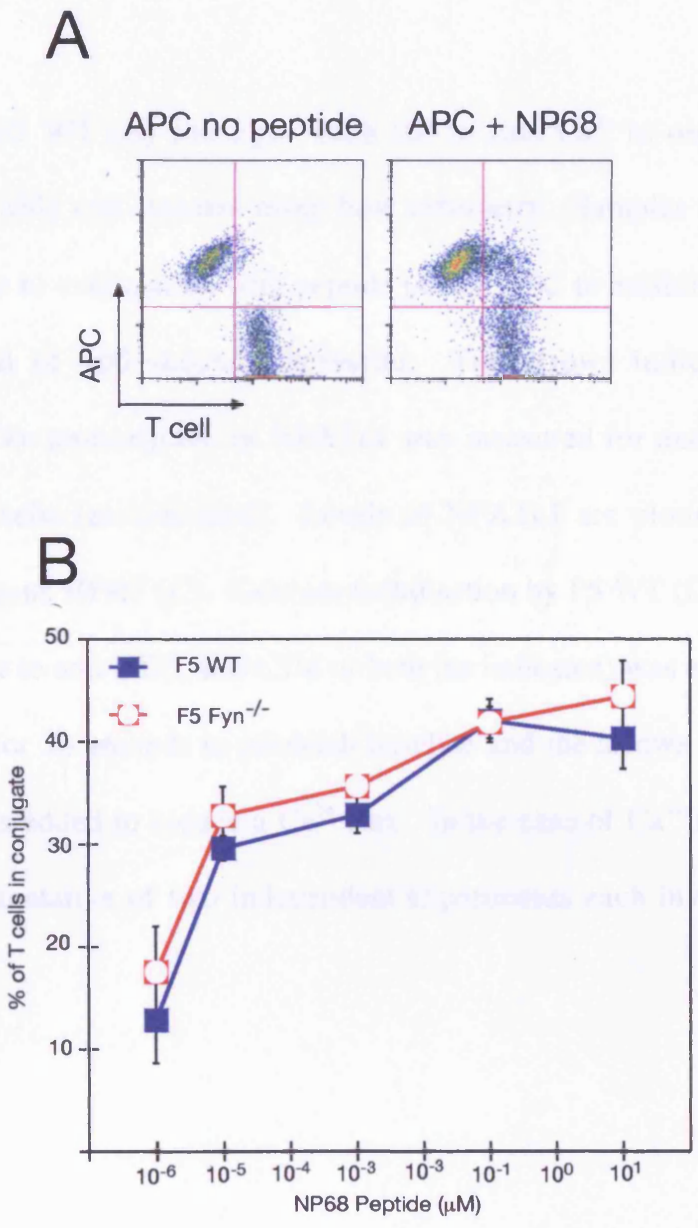

A
APC no peptide
APC + NP68
APC
T cell
B
F5 WT
F5 Fyn-/-
% of T cells in conjugate
50
40
30
20
10
10-6
10-5
10-4
10-3
10-2
10-1
100
101
NP68 Peptide (μM)

Figure 4.6: Fyn deficiency does not alter intracellular Ca^{2+} mobilisation in response to pMHC

The ability of F5 WT (A) and $Fyn^{-/-}$ cells (B) to flux Ca^{2+} in response to peptide pulsed APC (1 μM) was assesses using flow cytometry. Samples were acquired for 30 seconds prior to conjugation with peptide pulsed APC to establish a baseline, and then for a total of 400 seconds thereafter. The arrows indicate the point of stimulation. The transcription of NFATc1 was measured for antigen activated F5 WT and $Fyn^{-/-}$ cells (as indicated). Levels of NFATc1 are plotted relative to the house keeping gene HPRT (C). Calcium mobilisation by F5 WT (D) and $Fyn^{-/-}$ (E) T cells in response to anti-CD3, anti-CD8 or both (as indicated) was assessed. Samples were acquired for 30 seconds to establish baseline and the arrows indicate the point were avidin was added to induce a Ca^{2+} flux. In the case of Ca^{2+} mobilisation data, these are representative of two independent experiments each involving triplicated repeats.

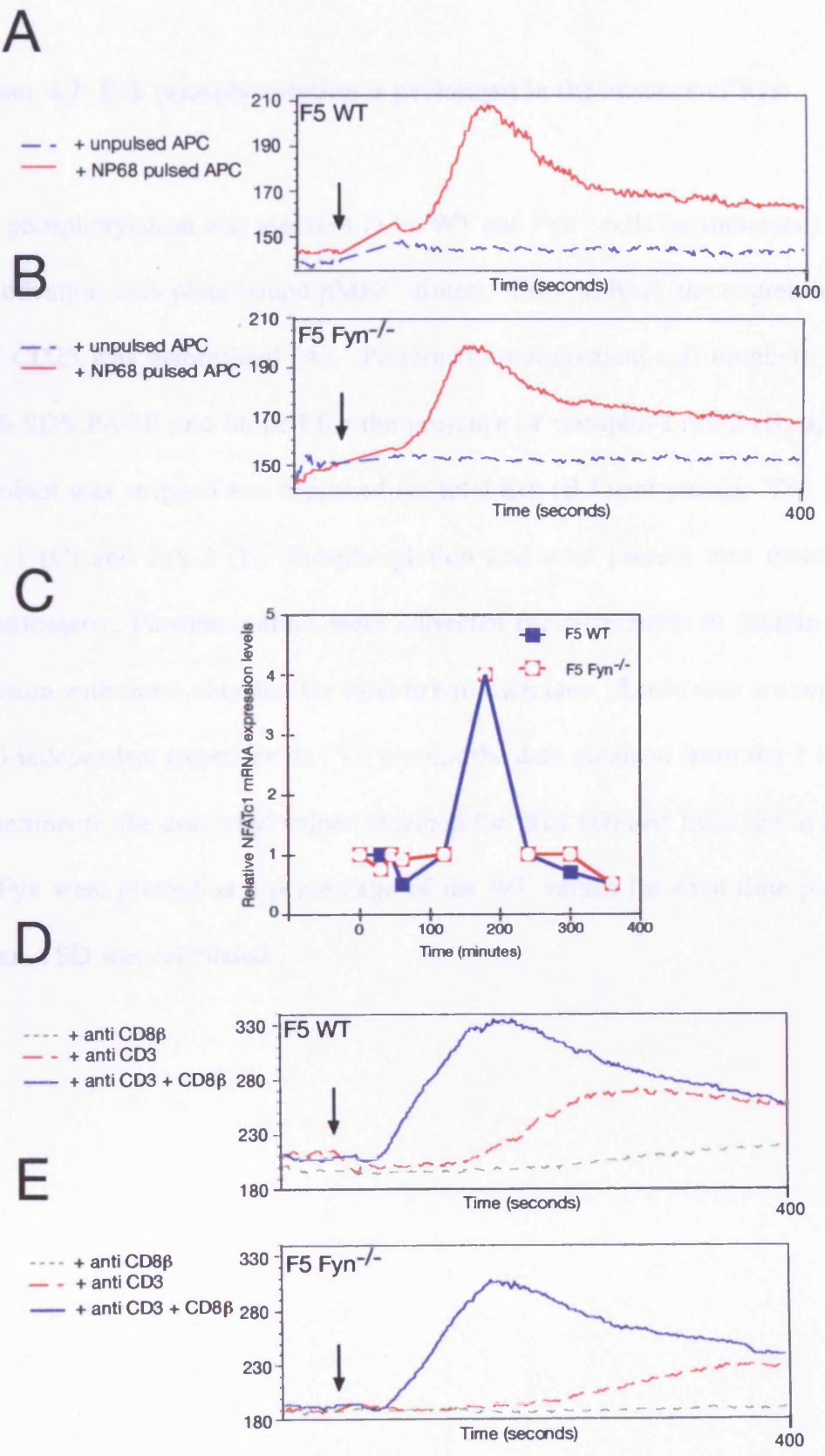
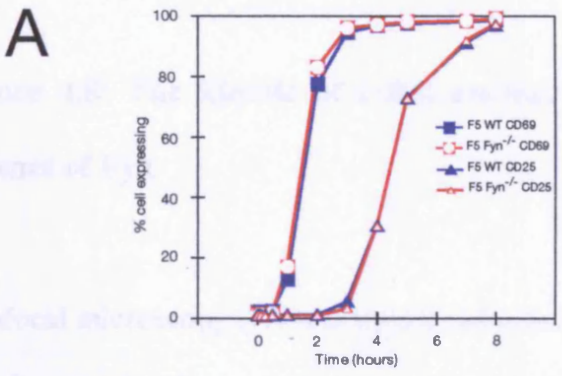
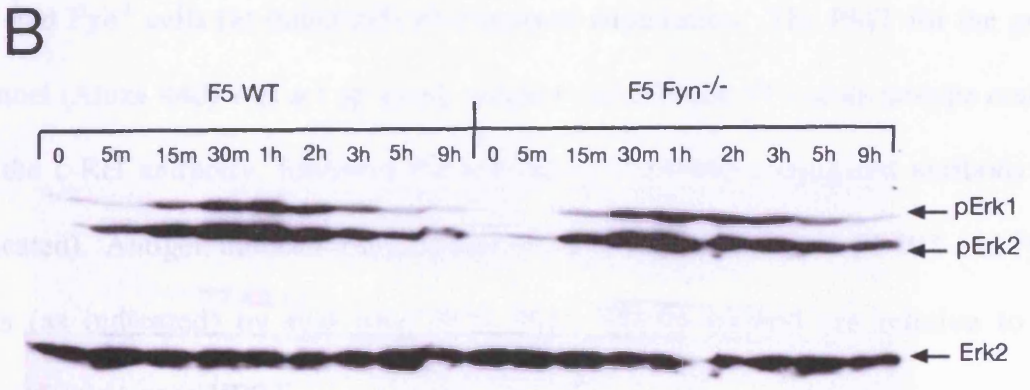

A
+ unpulsed APC
+ NP68 pulsed APC
F5 WT
210
190
170
150
Time (seconds)
400
B
+ unpulsed APC
+ NP68 pulsed APC
F5 Fyn-/-
210
190
170
150
Time (seconds)
400
C
Relative NFATc1 mRNA expression levels
F5 WT
F5 Fyn-/-
5
4
3
2
1
0
0
100
200
300
400
Time (minutes)
D
+ anti CD8β
+ anti CD3
+ anti CD3 + CD8β
F5 WT
330
280
230
180
Time (seconds)
400
E
+ anti CD8β
+ anti CD3
+ anti CD3 + CD8β
F5 Fyn-/-
330
280
230
180
Time (seconds)
400

Figure 4.7: Erk phosphorylation is prolonged in the absence of Fyn

Erk phosphorylation was assessed in F5 WT and $Fyn^{-/-}$ cells (as indicated) in response to activation with plate bound pMHC dimers. Prior to lysis, the expression of CD69 and CD25 was determined (A). Protein from equivalent cell numbers was run on 10% SDS-PAGE and blotted for the presence of phospho-Erk1/2 (B, upper panel). The blot was stripped and reprobed for total Erk (B lower panel). The intensity of Erk-1 (C) and Erk-2 (E) phosphorylation and total protein was measured using densitometry. Phospho-values were corrected for differences in protein loading by division with those obtained for total Erk in each lane. These data are representative fo 3 independent experiments. To present the data obtained from the 3 independent experiments, the corrected values obtained for Erk1 (D) and Erk2 (E) in the absence of Fyn were plotted as a percentage of the WT values for each time point and the mean ± SD was calculated.


A
100
80
60
40
20
0
% cell expressing
0
2
4
6
8
Time (hours)
F5 WT CD69
F5 Fyn-/- CD69
F5 WT CD25
F5 Fyn-/- CD25


B
F5 WT
F5 Fyn-/-
0 5m 15m 30m 1h 2h 3h 5h 9h 0 5m 15m 30m 1h 2h 3h 5h 9h
pErk1
pErk2
Erk2

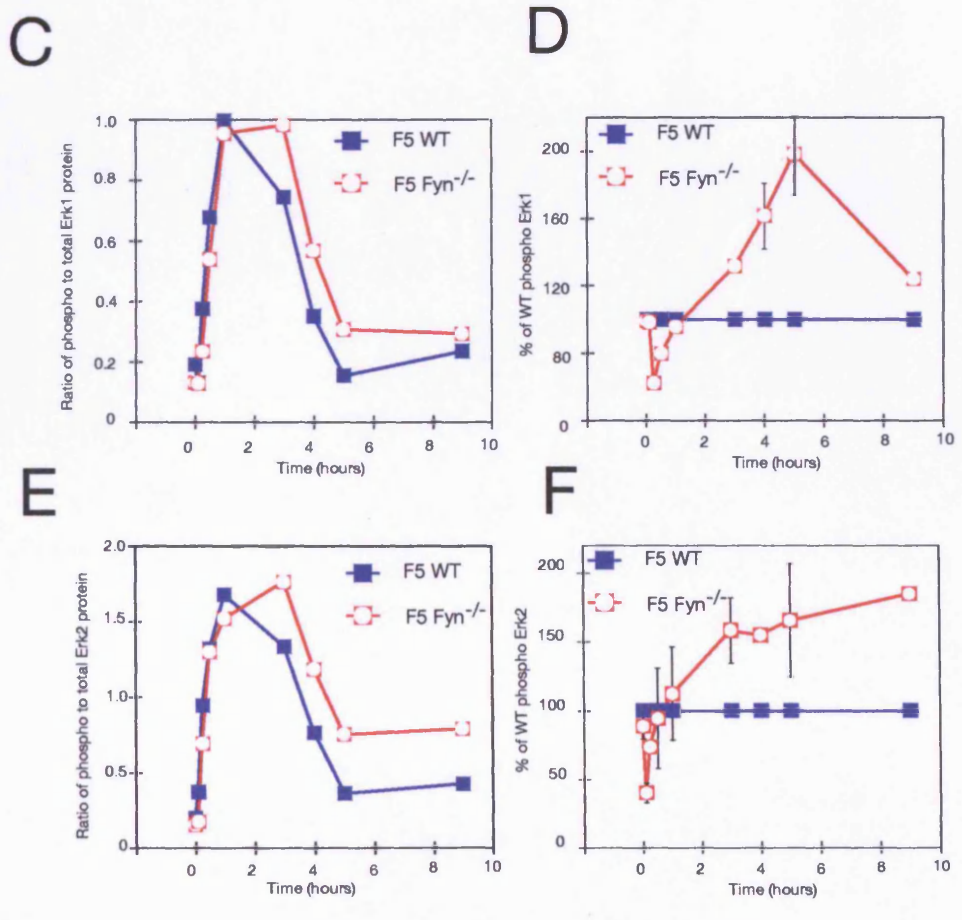
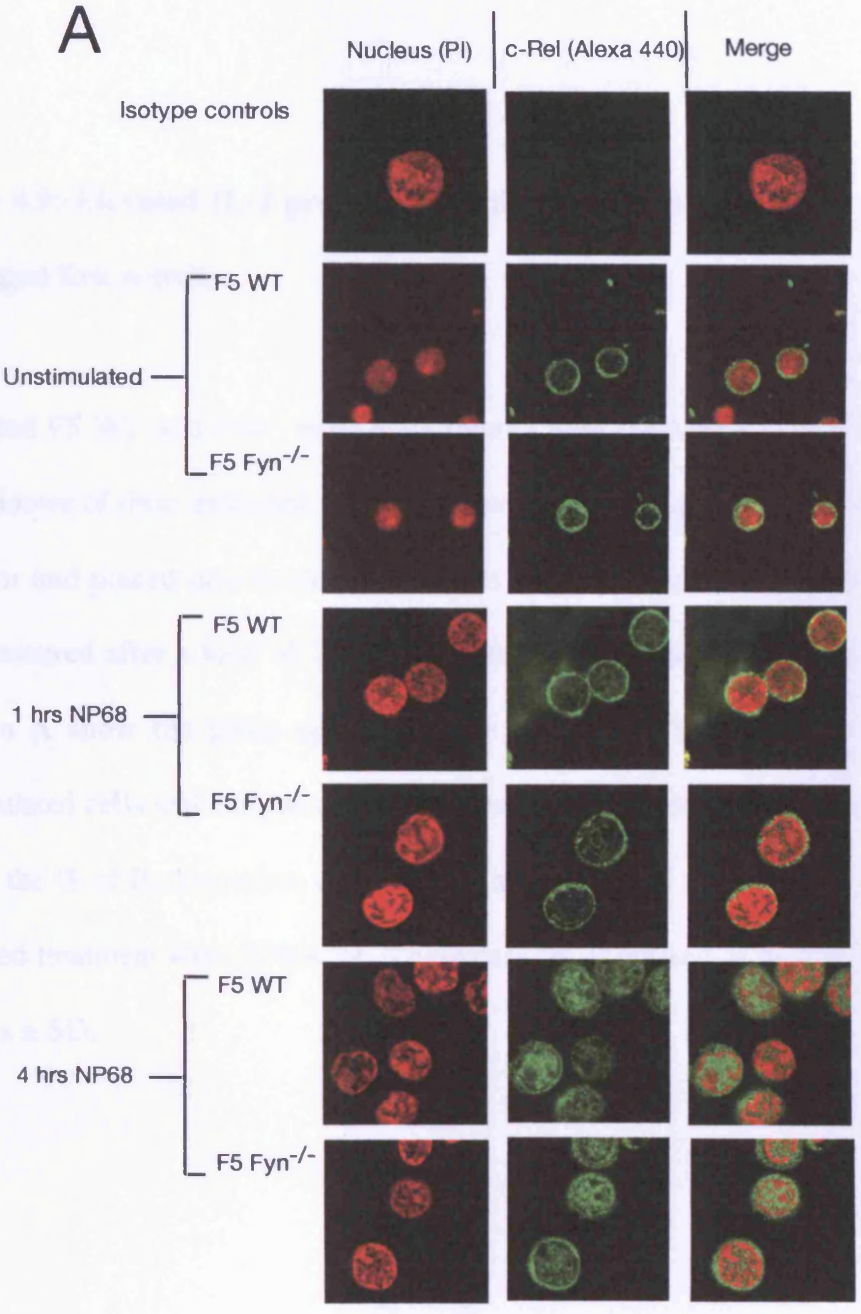

C
D
E
F
Ratio of phospho to total Erk1 protein
% of WT phospho Erk1
Ratio of phospho to total Erk2 protein
% of WT phospho Erk2
Time (hours)
F5 WT
F5 Fyn-/-

Figure 4.8: The kinetic of c-Rel nuclear translocation is unchanged in the absence of Fyn

Confocal microscopy (A) was used to address the nuclear translocation of c-Rel in F5 WT and $Fyn^{-/-}$ cells (as indicated) after antigen stimulation. The PMT for the green channel (Alexa 440) was set up using samples stained with PI and an isotype control for the c-Rel antibody, followed the secondary Alex-440 conjugated antibody (as indicated). Antigen induced transcription of c-Rel was measured in F5 WT and $Fyn^{-/-}$ cells (as indicated) by real time PCR (B). Values plotted are relative to the housekeeping gene HPRT.


A
Nucleus (PI)
c-Rel (Alexa 440)
Merge
Isotype controls
Unstimulated
F5 WT
F5 Fyn-/-
1 hrs NP68
F5 WT
F5 Fyn-/-
4 hrs NP68
F5 WT
F5 Fyn-/-

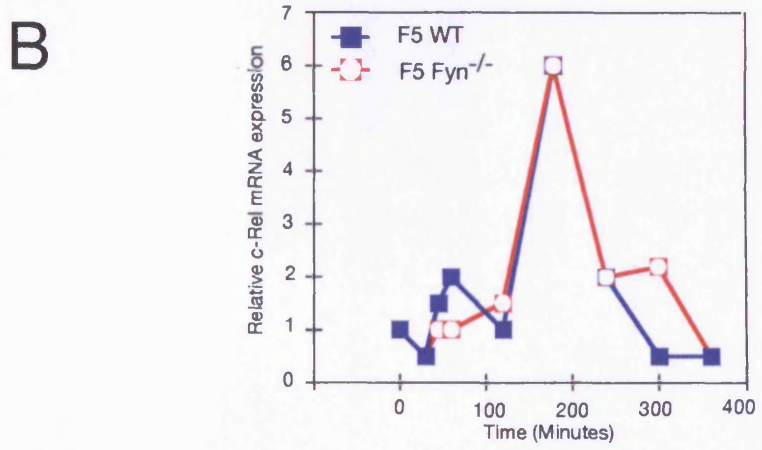
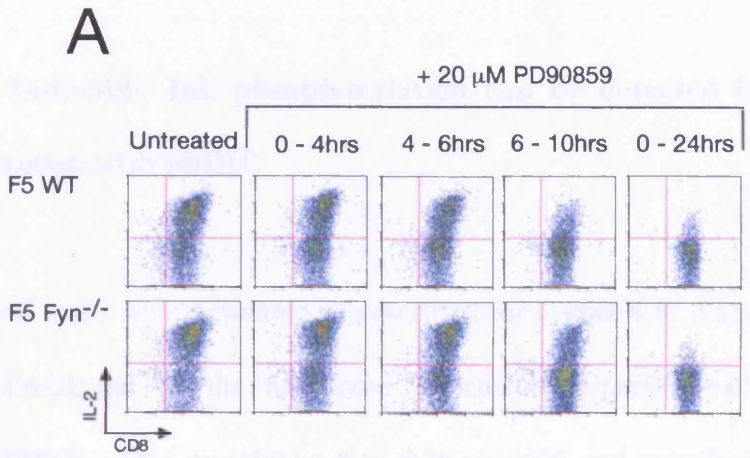
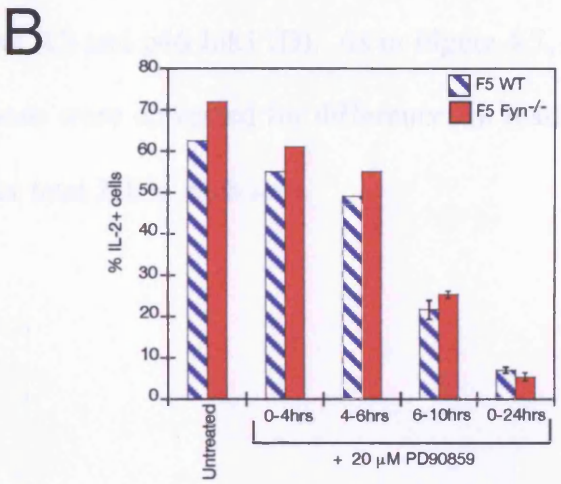

B
F5 WT
F5 Fyn-/-
Relative c-Rel mRNA expression
0
1
2
3
4
5
6
7
0
100
200
300
400
Time (Minutes)

Figure 4.9: Elevated IL-2 production in the absence of Fyn is not solely due to prolonged Erk activity.

Activated F5 WT and $Fyn^{-/-}$ cells were treated with the Mek inhibitor PD90859 for the windows of time indicated. Cells were washed thoroughly to remove any trace of inhibitor and placed into fresh culture wells with stimulus. The production of IL-2 was measured after a total of 24 hrss of culture by intracellular staining. The FACS plots in A show the gates applied to the samples. These gates were set using unstimulated cells and isotype control antibodies. These data were used to generate a plot of the % of IL-2 positive cells (B) and the MFI of IL-2 production (C) after the indicated treatment with PD90859. These data are expressed as the mean of triplicate samples ± SD.


A
+ 20 μM PD90859
Untreated
0 - 4hrs
4 - 6hrs
6 - 10hrs
0 - 24hrs
F5 WT
F5 Fyn-/-
IL-2
CD8


B
F5 WT
F5 Fyn-/-
% IL-2+ cells
80
70
60
50
40
30
20
10
0
Untreated
0-4hrs
4-6hrs
6-10hrs
0-24hrs
+ 20 μM PD90859

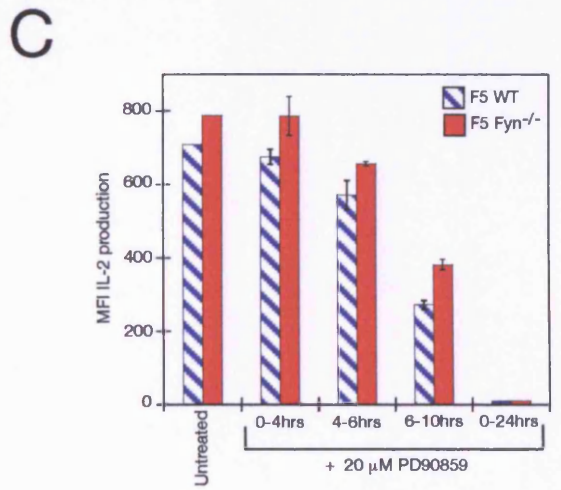

C
F5 WT
F5 Fyn-/-
MFI IL-2 production
800
600
400
200
0
Untreated
0-4hrs
4-6hrs
6-10hrs
0-24hrs
+ 20 μM PD90859

Figure 4.10: Inducible Jnk phosphorylation can be detected in $Fyn^{-/-}$ cells following activation with pMHC

F5 WT and $Fyn^{-/-}$ cells were activated as described for Figure 4.8. Samples were run out on a 10% PAGE gel and the membrane blotted for the presence of phospho-Jnk 1/2.(A, upper panel). This membrane was then stripped and reprobed for total Jnk protein (A, lower panel). Densitometry was then used to quantify the levels of p54 Jnk 1/2 (B), p46 Jnk2 (C) and p46 Jnk1 (D). As in Figure 4.7, the values obtained for the phospho Jnk bands were corrected for differences in loading by dividing by the numbers obtained for total Jnk in each lane.

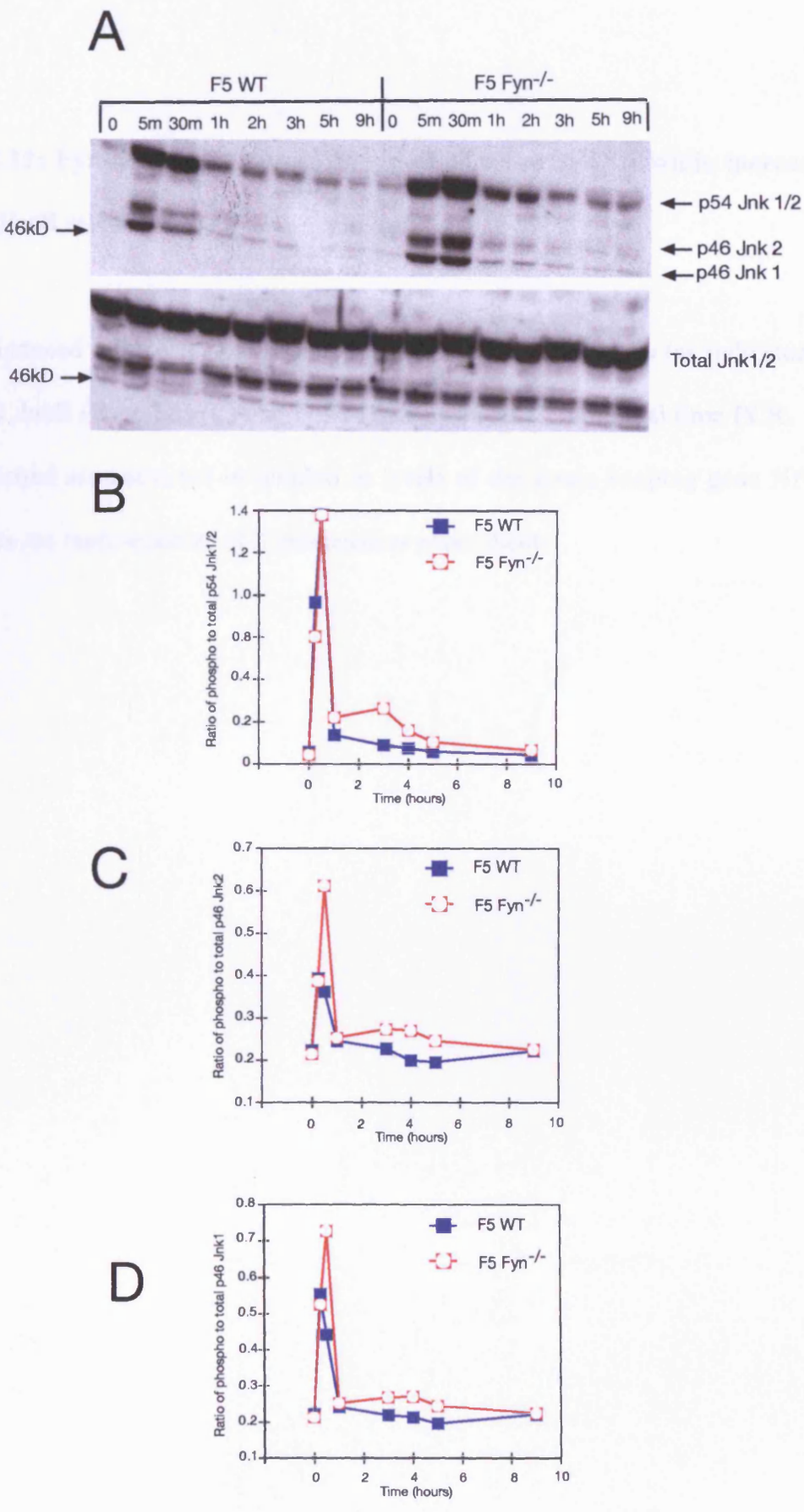
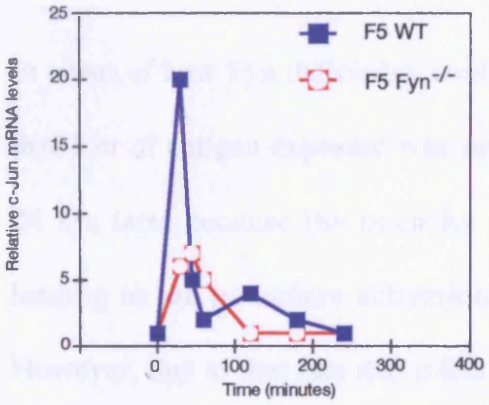

A
F5 WT
F5 Fyn-/-
0 5m 30m 1h 2h 3h 5h 9h 0 5m 30m 1h 2h 3h 5h 9h
46kD
p54 Jnk 1/2
p46 Jnk 2
p46 Jnk 1
46kD
Total Jnk1/2
B
Ratio of phospho to total p54 Jnk1/2
F5 WT
F5 Fyn-/-
Time (hours)
C
Ratio of phospho to total p46 Jnk2
F5 WT
F5 Fyn-/-
Time (hours)
D
Ratio of phospho to total p46 Jnk1
F5 WT
F5 Fyn-/-
Time (hours)

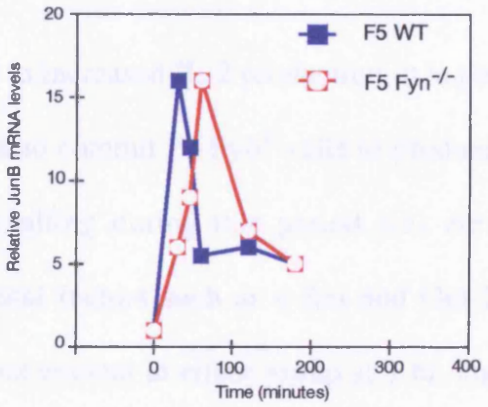
Figure 4.11: Fyn deficiency lowers the level of c-Jun mRNA while increasing levels of FosB mRNA

Antigen induced mRNA levels by activated F5 WT and $Fyn^{-/-}$ cells (as indicated) of c-Jun (A), JunB (B), c-Fos (C) and FosB (D) was assessed using real time PCR. The values plotted are corrected in relation to levels of the house-keeping gene HPRT. These data are representative of 2 independent experiments.

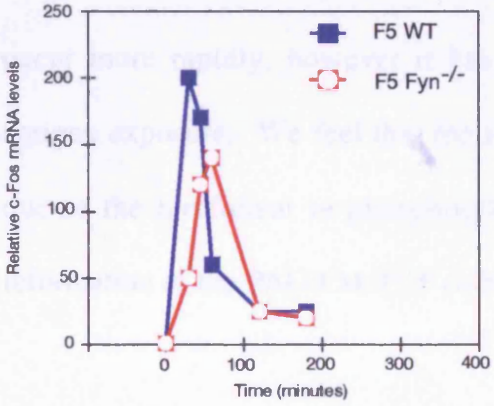
A


F5 WT
F5 Fyn-/-
Relative c-Jun mRNA levels
Time (minutes)
0
5
10
15
20
25
0
100
200
300
400

B


F5 WT
F5 Fyn-/-
Relative JunB mRNA levels
Time (minutes)
0
5
10
15
20
0
100
200
300
400

C


F5 WT
F5 Fyn-/-
Relative c-Fos mRNA levels
Time (minutes)
0
50
100
150
200
250
0
100
200
300
400

D

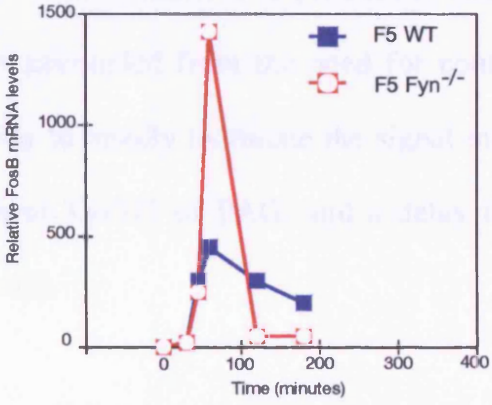
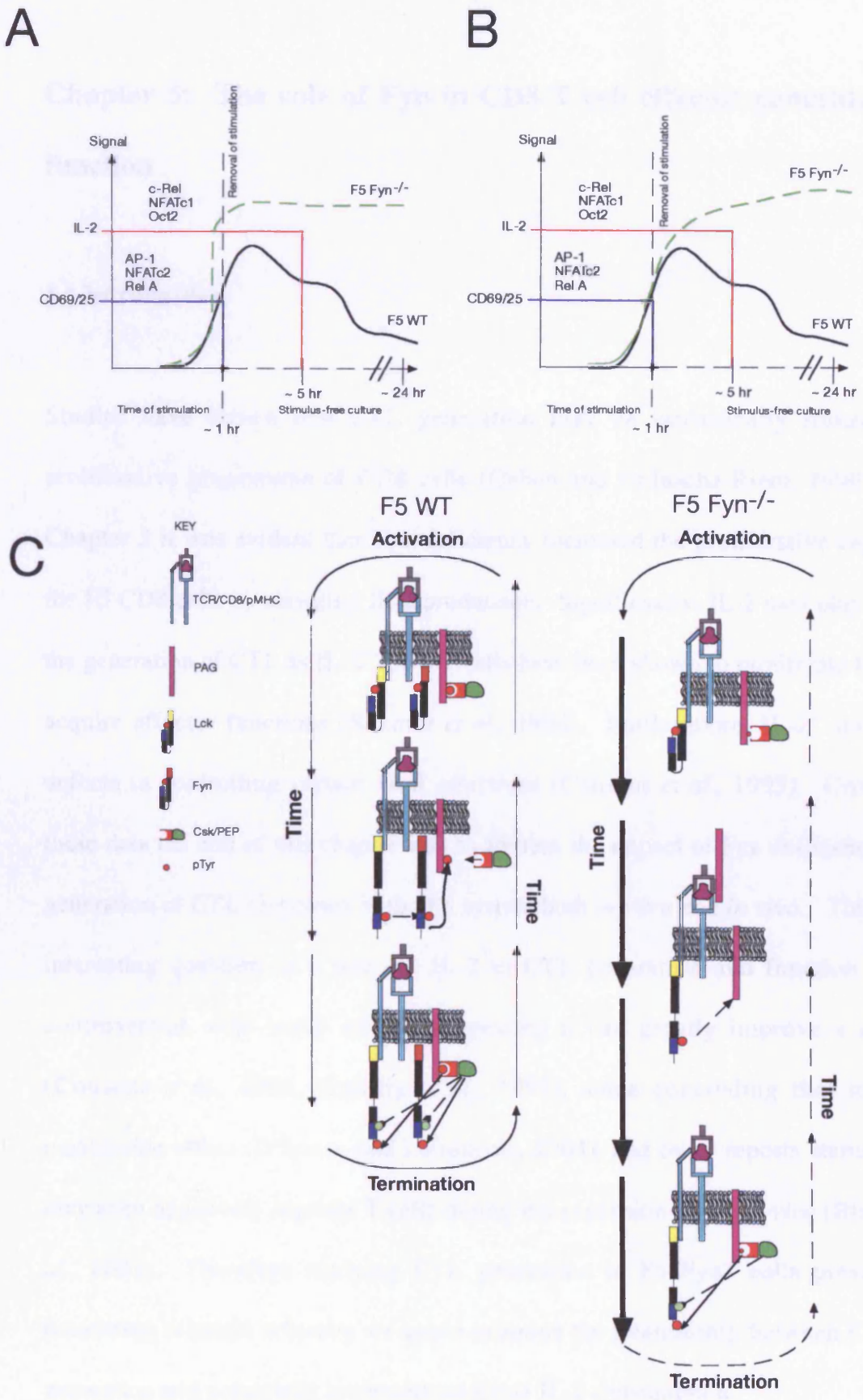

F5 WT
F5 Fyn-/-
Relative FosB mRNA levels
Time (minutes)
0
500
1000
1500
0
100
200
300
400

Figure 4.12: A possible mechanism for how Fyn deficiency could be affecting the ability of F5 cells to produce IL-2

In terms of how Fyn deficiency could lead to increased IL-2 production, it is possible that 1 hr of antigen exposure was sufficient to commit F5 $Fyn^{-/-}$ cells to produce IL-2 24 hrs later because the intensity of signalling during this period was elevated, leading to the premature activation of distal factors such as c-Rel and Oct-2 (A). However, due to that fact that c-Rel was not evident in either group at 1 hr, and that IL-2 production 24 hrs later remained sensitive to Src inhibition 3 hrs after activation, this first model may be unlikely. The second model suggest that upon removal of stimulus, F5 WT cells terminate signalling and never reach the required threshold for IL-2 production, however F5 $Fyn^{-/-}$ cells continue to signal even in the absence of antigen and are able to produce IL-2 (B). In this model, IL-2 production does not occur more rapidly, however it has been uncoupled from the need for continual antigen exposure. We feel that the inability to rapidly terminate the signal may be due to the inefficient re-phosphorylation of Tyr317 of PAG, and a delay in the reformation of the PAG/Csk/PEP complex (C).


A
B
Signal
Removal of stimulation
c-Rel
NFATc1
Oct2
F5 Fyn-/-
IL-2
AP-1
NFATc2
Rel A
CD69/25
F5 WT
~ 5 hr
~ 24 hr
Time of stimulation
~ 1 hr
Stimulus-free culture
C
KEY
TCR/CD8/pMHC
PAG
Lck
Fyn
Csk/PEP
pTyr
F5 WT
F5 Fyn-/-
Activation
Time
Termination

Chapter 5: The role of Fyn in CD8 T cell effector generation and function

5.1 Introduction

Studies have shown that CTL generation may be intrinsically linked to the proliferative programme of CD8 cells (Oehen and Brduscha-Riem, 1998). From Chapter 3 it was evident that Fyn deficiency increased the proliferative capacity of the F5 CD8 cells by elevating IL-2 production. Significantly, IL-2 may play a role in the generation of CTL as IL-$2^{-/-}$ CD8 T cells have been shown to proliferate but not to acquire effector functions (Kramer *et al.*, 1994). Furthermore, IL-$2^{-/-}$ mice show defects in controlling certain viral infections (Cousens *et al.*, 1995). Considering these data the aim of this chapter was to address the impact of Fyn deficiency on the generation of CTL responses in the F5 system both *in vitro* and *in vivo*. This was an interesting question as a role for IL-2 in CTL generation and function remains controversial, with some studies suggesting it can greatly improve a response (Cousens *et al.*, 1995; Kundig *et al.*, 1993), some concluding that it has no measurable effect (D'Souza and Lefrancois, 2004), and other reports stating that it may even negatively regulate T cells during the expansion phase *in vivo* (Blattman *et al.*, 2003). Therefore studying CTL generation in F5 $Fyn^{-/-}$ cells presented an interesting scenario whereby we could examine the relationship between CD8 CTL generation and potentially increased autocrine IL-2 consumption.

In a naïve mouse, antigen-specific CD8 T cell clones are present at extremely low frequencies; however, on encountering antigen they undergo a rapid clonal expansion and differentiate into a CTL. The function of a CTL is to identify and then eliminate cells expressing specific antigen in the context of MHC-class I molecules. Lysis of a target cell by a CTL is achieved by mechanisms that require the *de novo* expression of molecules not present in a naïve CD8 T cell (Kaech *et al.*, 2002). These include IFNγ (Billiau, 1996) and lytic granules such as Granzyme B and Perforin (Trapani *et al.*, 2000; Kagi *et al.*, 1996). Therefore, activation of naïve CD8 T cells leads transcription of these effector molecules allowing for the elimination of viral infections to occur (Harty *et al.*, 2000). There are two main pathways responsible for removal of a potentially infected cell by CTL. One involves the production and secretion of lytic molecules that include Perforin and Granzymes: the other involves the FasL pathway and the triggering of apoptosis through death receptors (Suda and Nagata, 1994). Interestingly, CTL are often termed as "serial killers", as after releasing the primary store of lytic granules, they can resynthesis Perforin and Granzyme in order to then target further cells for lysis (Isaaz *et al.*, 1995).

In terms of the lytic mechanisms, Perforin functions by undergoing a polymerisation reaction and inserting itself into the plasma membrane of a target cell to form pores, thus allow solutes and small ions to enter the cytoplasm (Podack and Konigsberg, 1984). CTL from mice deficient in Perforin are unable to carry out any granule-mediated killing (Kagi *et al.*, 1994), and are therefore severely immuno-compromised. Furthermore, studies have shown that the promoter of the Perforin

gene contains at least two STAT5 binding sites (Yu *et al.*, 1999; Zhang *et al.*, 1999) making expression sensitive to IL-2 receptor signals.

As granule mediated cytolysis was also shown to be able to induce apoptosis, it was interesting that Perforin induced membrane damage but not DNA laddering (Duke *et al.*, 1983). This observation led to the discovery of further lytic molecules including Granzyme B (Lobe *et al.*, 1986) that could synergise with Perforin and pass through the pores to initiate apoptosis by activating Caspase 3 (Darmon *et al.*, 1995). Granzyme B knockout cells are unable to mediate CTL driven DNA damage (Heusel *et al.*, 1994), however they are not as severely affected as Perforin deficient mice, highlighting the fact that Perforin expression is probably the rate-limiting step in a CTL response. As for Perforin gene regulation, there is evidence that IL-2 signalling is able to regulate the expression of Granzyme B in T cells and NK cells, however the direct mechanism for this at the transcriptional level is currently unknown (DeBlaker-Hohe *et al.*, 1995; Manyak *et al.*, 1989).

Activation of naïve CD8 T cells also induces the expression of the cytokine IFNγ, which can improve CTL responses by inducing the upregulating of MHC class I expression, increasing immune recognition of the target cell and by suppressing viral replication. IFNγ may also be important for downmodulating an immune response as $IFN\gamma^{-/-}$ mice possess increased CD8 cell numbers during the expansion and contraction phases following viral challenge (Badovinac *et al.*, 2000). Furthermore, while $IFN\gamma^{-/-}$ mice show defects in dealing with *Listeria monocytogenes* and vaccinia

virus, they are still able to mount a granule-mediated CTL response (Lu *et al.*, 1998; Huang *et al.*, 1993) suggesting that the lytic killing machinery can work independently of IFNγ. Moreover, there is also evidence that IL-2 can regulate production of IFNγ in a CTL response (Farrar *et al.*, 1985; Reem and Yeh, 1984; Kasahara *et al.*, 1983). However it is not known how this is achieved but may be due to the presence of a STAT5 binding element in the promoter of the *IFNγ* gene (Bream *et al.*, 2004)

As well as granule mediated cytotoxicity, CD8 cells also induce apoptosis in target cells through the production of FasL (CD154). FasL is expressed in a membrane bound form after T cell activation (Anel *et al.*, 1994; Vignaux and Golstein, 1994; Suda *et al.*, 1993) and is then able to bind to the Fas receptor on the surface of other target cells and can induce apoptosis (Suda and Nagata, 1994). However, this mechanism is thought to be preferential for the removal of activated T cells by AICD, and may be less important to the killing of virally infected targets. There is also some evidence that IL-2 can influence the expression of FasL (Ye *et al.*, 1996).

Using the F5 system it is possible to look at the generation and function of CTL both *in vitro* and *in vivo*. For *in vitro* studies, it is possible to activate F5 cells using the NP68 antigen and look at the expression of effector molecules. Furthermore, the ability of these *in vitro* generated CTL to lyse NP68 pulsed target cells can be addressed using the classical killing assay (Kramer *et al.*, 1994). Mice expressing the F5 TCR transgene will also respond to administration of the A/NT/60-68 strain of

influenza (Moskophidis and Kioussis, 1998) making it possible to look at CTL generation and function *in vivo*.

5.2 F5 and polyclonal Fyn$^{-/-}$ CD8 cells produce more IFNγ than WT control cells

We began addressing the generation of CTL responses in the absence of Fyn by determining the ability of F5 cells to commit to IFNγ production using intracellular staining. Figure 5.1A and 5.1B show that 24 hrs after NP68 stimulation, and following a 4 hr recall stimulus with PdbU and ionomycin, both F5 Fyn$^{-/-}$ and F5 WT cells had identical potential to produce IFNγ. At 48 and 72 hrs, F5 Fyn$^{-/-}$ T cells continued to produce this cytokine at similar levels to that observed at 24 hrs, in comparison production by F5 WT cell declined by 4-fold at these times. Moreover, F5 Fyn$^{-/-}$ cells continued to produce elevated levels of IFNγ compared to F5 WT cells at each round of division.

As previously mentioned with respect to IL-2 measurement, because intracellular cytokine staining is only visible after restimulation with PdbU and ionomycin it provides information on locus accessibility rather than what the cell is actually producing at that time. To determine the influence Fyn deficiency was having on IFNγ production after primary activation we measured IFNγ mRNA levels using real time PCR. From figure 5.1C it is evident that maximal IFNγ mRNA was detected in WT F5 cells at 10 hrs whereas levels in activated F5 Fyn$^{-/-}$ cells peaked at 5 hrs. Moreover, maximal production of IFNγ mRNA by Fyn deficient F5 T cells was ~7-

fold higher than by WT F5 cells. At 24 hrs IFNγ mRNA was still elevated 50-fold compared to the housekeeping gene HPRT, but it was undetectable in WT F5 cells at this time. Comparing these data to the data in Figure 5.1A there is a striking difference. In F5 WT cells it is clear that intracellular IFNγ could be measured at 24 hrs after 4 hrs PdbU and ionomycin treatment, but this did not correlate with mRNA levels as they were undetectable in WT cells at this time. This difference was most probably due to the methods employed and the need for restimulation to detect intracellular IFNγ protein.

In Chapter 3 we determined that polyclonal $Fyn^{-/-}$ cells behaved like antigen-activated F5 $Fyn^{-/-}$ cells after stimulation with anti-CD3 and anti-CD8 coated beads. Therefore we addressed the ability of purified naive polyclonal $Fyn^{-/-}$ CD8 cells to produce IFNγ by intracellular cytokine staining after activation with anti-CD3/CD8 coated microbeads. Figure 5.1D shows that at 72 hrs after stimulation, Fyn deficient cells were producing ~1.5-fold more IFNγ on a per-cell basis than purified naive CD8 cells from WT polyclonal mice. Collectively these data suggest that IFNγ production was elevated in the absence of Fyn in both monoclonal and polyclonal cells.

5.3 F5 $Fyn^{-/-}$ cells show enhanced CTL activity *in vitro*

Next we wanted to measure the cytotoxic activity of the *in vitro* generated CTL using the classical killing assay. After the 3-day activation and culture period required to generate CTL, we phenotyped the cells before inclusion in the assay. Specifically we

looked at cell size, and CD44 expression using FACS (figure 5.2A) to check that both groups of cells had activated comparably. It is clear from the data in figure 5.2A that after three days stimulation an equivalent number of cells had been activated as judged by increase in cell size (Fsc) and CD44 expression, however the MFI of CD44 was reduced in the absence of Fyn. There were also equivalent frequencies of cells within the live gate (data not shown).

To further determine the efficiency of CTL generation after the 3-day culture period we measured intracellular Granzyme B levels. Unlike intracellular IFNγ protein detection, this did not require any restimulation with PdbU and ionomycin. Figure 5.2B shows that 100% of cells in both groups were expressing Granzyme B, however the level of production was elevated in the absence of Fyn on a per-cell basis (~2000 MFI units in WT F5, compared to ~3000 MFI units in F5 $Fyn^{-/-}$ cells). Interestingly, CD5 expression, like CD44, was marginally reduced in the absence of Fyn compared to F5 WT cells.

Prior to inclusion in the CTL assay, cells were counted and adjusted so that equal numbers of blasting cells were incubated with a fixed number of target cells in each group. From the data presented in figure 5.2C it is clear that, at all but the highest effector to target ratios tested, F5 $Fyn^{-/-}$ CTL were ~27 times more efficient in antigen-specific killing than F5 WT CTL on a per-cell basis.

5.4 The upregulation of CD44 and CD5 is reduced in the absence of Fyn

Having shown that the expression of CD5 and CD44 was reduced on *in vitro* generated $Fyn^{-/-}$ CTL after 3 days of culture, we wanted to look at the upregulation of these molecules over a range of antigen doses at 24 hrs. CD44 is thought to reflect the activation status of a T cell and also considered to be a marker of memory CD8 T cells (Kaech *et al.*, 2002), whereas CD5 is considered to be a negative regulator of T cell activation (Tarakhovsky *et al.*, 1995). Figures 5.3A and B shows that over the range NP68 concentrations used, the ability of F5 $Fyn^{-/-}$ cells to upregulate CD44 was consistently reduced compared to F5 WT cells as measured by the MFI of CD44 expression. In contrast the overall percentage of $CD44^{+}$ cells remained comparable. The same observation was made when CD5 upregulation was assessed (Figure 5.3C and D). Interestingly, as noted in Chapter 3, the expression of molecules such as CD69 and CD25 was comparable at 24 hrs in both groups (data not shown). The fact that CD44 expression and CD5 expression have been shown to increase during the transition of naïve CD8 cells to a memory cell (Kaech *et al.*, 2002) could reflect possible differences in the progression of F5 $Fyn^{-/-}$ cells along this developmental path.

5.5 Elevated Granzyme B production by F5 Fyn$^{-/-}$ cells is driven by increased IL-2 levels.

In F5 CD8 T cells lacking Fyn, activation using NP68 peptide led to increased IL-2 production and increased effector generation / function. The next set of experiments was designed to ask whether the two observations were in fact linked, or whether they were distinct phenomena. IL-2 has been suggested to play a role in the generation of F5 CD8 effector function (Kramer *et al.*, 1994); therefore interesting to assess the impact that the increased IL-2 production by Fyn$^{-/-}$ cells may have on the generation of CTL. To this end, the strategy of blocking IL-2 and spiking with IL-2 was employed as described for Chapter 3, Figure 3.6.

Firstly, Granzyme B protein was measured in both groups after IL-2 manipulation *in vitro*. As previously mentioned, IL-2 can modulate the expression of the Granzyme B in T cells (DeBlaker-Hohe *et al.*, 1995; Manyak *et al.*, 1989). From Figure 5.4A it is clear that blocking IL-2 reduced the frequency of Granzyme B positive cells in the Fyn$^{-/-}$ group at 48 and 72 hrs to that of unmanipulated WT F5 cell levels. Blocking IL-2 with S4B6 lowered the percentage of Granzyme B expressing F5 WT cells also. Figure 5.4B shows that spiking cultures with IL-2 increased the frequency of Granzyme B positive cells in F5 WT samples to that of unmanipulated Fyn deficient samples, both at 48 and 72 hrs. IL-2 manipulation also influenced the MFI of Granzyme B expression in the same manner (data not shown). As described for CD25 expression (Chapter 3, Figure 3.6), addition of excess IL-2 to Fyn deficient

cells was unable to elevate expression of Granzyme B compared to non-treated $Fyn^{-/-}$ cells suggesting that the response to IL-2 was already maximal.

Interestingly, while we could clearly influence Granzyme B expression at 48 and 72 hrs, the addition of recombinant IL-2 had no effect on WT F5 cells at 24 hrs, neither did blocking antibody have an effect on F5 $Fyn^{-/-}$ cells at this time. This suggests that either the various IL-2 treatments took time to exert an effect, or that Granzyme B expression at 24 hrs is IL-2 independent. If it was the latter reason, then this suggest that F5 $Fyn^{-/-}$ cells are able to make more Granzyme B at 24 hrs independently of IL-2 levels. It is possible that the differences in the AP-1 members noted in Chapter 4 could exert an effect on the Granzyme promoter as it contains an AP-1 site, however it is a consensus sequence (Babichuk *et al.*, 1996) and may not be sensitive to changes in Fos-Jun components.

5.6 Elevated IFNγ production by F5 $Fyn^{-/-}$ is driven by increased IL-2 production.

Next we assessed the impact of IL-2 availability on the ability of F5 $Fyn^{-/-}$ to produce more IFNγ after activation. A number of studies have suggested that IL-2 can regulate IFNγ expression (Farrar *et al.*, 1985; Reem and Yeh, 1984; Kasahara *et al.*, 1983). Figure 5.5A shows that blocking with S4B6 reduced production of this cytokine by $Fyn^{-/-}$ cells to that of unmanipulated WT F5 cells at 72 hrs. The reciprocal experiment of spiking cultures with IL-2 was able to induce F5 WT cells to

acquire the potential to produce ~2 fold more IFNγ at 48 hrs than Fyn deficient cells without manipulation (Figure 5.5B). Therefore F5 WT cells had the capacity to produce large quantities of IFNγ, however this was clearly dependent on IL-2 availability. In both groups, the percentage of cells able to make IFNγ was ~90% (data not shown). As noted previously for Granzyme B expression, addition of IL-2 to $Fyn^{-/-}$ cells did not elevate levels above untreated controls, again suggesting an already maximal response to this cytokine.

5.7 Activation of F5 $Fyn^{-/-}$ T cells *in vivo* leads to elevated IL-2 / IFNγ production and increased cell recoveries.

Next we wanted to address whether the improved generation of CTL in the absence of Fyn would also occur *in vivo*. As mentioned previously, the F5 TCR is specific for the NP68 peptide derived from the nuclear coat protein of the A/NT/60-68 strain of the influenza virus (Mamalaki *et al.*, 1993; Mamalaki *et al.*, 1992; Townsend *et al.*, 1985), therefore, it was possible to use live virus to activate F5 cells. However we wanted to activate cells *in vivo* without allowing the virus to replicate in lung tissue, which could be fatal to the host. Instead we transferred 3 x 10^6 F5 T cells with virus i.v. into Rag-$1^{-/-}$ recipient mice, as the virus would not be pathogenic via this route. As shown in Figure 5.6A, F5 CD8 T cells transferred without virus did not show signs of activation after 7 days, as there was no upregulation of CD44. Therefore any proliferation could be solely attributed to activation by virus and not due to transferring cells into an empty host. From figure 5.6A it is clear that 7 days after

transfer with virus 100% of the F5 CD8 T cells became activated and upregulated CD44 in both the F5 $Fyn^{-/-}$ and F5 WT groups. However, as shown *in vitro*, the level of CD44 was reduced on the surface of F5 $Fyn^{-/-}$ cells compared to F5 WT controls. Cell recoveries and frequencies after 7 days were also assessed in the different peripheral lymphoid organs to give an indication of cell expansion. From Figure 5.6B and 5.6C it was clear that more F5 CD8 T cells were recovered from the lymph nodes and spleen of Rag-$1^{-/-}$ mice that had received flu virus and cells from F5 $Fyn^{-/-}$ donors compared to F5 WT controls. Furthermore, the frequency of CD8 PBL in the blood of the mice was also elevated in the absence of Fyn compared to WT controls (Figure 5.6D) However it seemed that the percentage of CD8 cells in the PEC was not grossly different (Figure 5.6E). It appears that both *in vitro* and *in vivo*, F5 $Fyn^{-/-}$ T cell proliferation and/or survival was increased after peptide activation.

When cells from the LN and spleen were restimulated *ex vivo* with PdbU and ionomycin (Figure 5.6F), a greater frequency of Fyn deficient cells were able to produce IL-2 (54% F5 WT, 77.5% F5 $Fyn^{-/-}$) and the MFI was also elevated compared to WT cells (88.5 MFI units in F5 WT, compared to 137 MFI units in F5 $Fyn^{-/-}$). Furthermore, analysis of IFNγ production by intracellular staining (Figure 5.6G) revealed that a higher percentage of virally activated $Fyn^{-/-}$ cells were able to produce this cytokine after restimulation compared to WT cells (58.2% F5 WT compared to 76.6 F5 $Fyn^{-/-}$). Collectively these data show that there was an increased potential to make IL-2 and IFNγ after *in vivo* activation, and that this correlated with

increased cell numbers recovered from the recipients of F5 Fyn$^{-/-}$ cells compared to WT controls.

4.8 Discussion

The aim of this chapter was to address the generation of CTL responses in the absence of Fyn, and determine if the increase in IL-2 production was affecting the differentiation programme of F5 Fyn$^{-/-}$ cells.

Production of IFNγ by F5 Fyn$^{-/-}$ cells after antigen activation was elevated compared to WT F5 cells as measured at the protein level by intracellular staining and at the mRNA level by real time PCR. Furthermore levels of Granzyme B protein were also increased, however CD5 and CD44 upregulation was reduced in the absence of Fyn. The increase in effector molecule levels translated into an improved CTL response *in vitro* by F5 Fyn$^{-/-}$ cells compared to WT controls. Furthermore, the increase in effector molecule expression was shown to be mediated by IL-2 as the IL-2 blocking antibody S4B6 reduced expression of IFNγ and Granzyme B by F5 Fyn$^{-/-}$ cells to that of untreated F5 WT cells. The reciprocal experiments of adding IL-2 was able to greatly improve the response of F5 WT cells. Finally, we showed that activation of F5 Fyn$^{-/-}$ cells *in vivo* using influenza virus also increased cell proliferation/survival and IL-2/IFNγ production compared to F5 WT cells.

Several studies have shown that CD8 CTL function is influenced by the presence of IL-2. For example infection of IL-$2^{-/-}$ mice using LCMV and vaccinia virus resulted in a 3-fold reduction in CTL activity (Cousens *et al.*, 1995; Kundig *et al.*, 1993). Also, in the F5 system, IL-$2^{-/-}$ CD8 cells did not become effector CTL (Kramer *et al.*, 1994). However, some studies have suggested that IL-2 has no effect on CTL generation. For example LeFrancois and colleagues infected WT or IL-$2^{-/-}$ mice with VSV and then addressed the CTL activity using an *in vitro* killing assay (D'Souza and Lefrancois, 2004). They observed no differences in the efficiency of killing peptide pulsed target cells by CTL generated in either WT or IL-$2^{-/-}$ mice, and concluded that IL-2 is not required for CTL function or generation. A possible reason for the differences between our data and that reported by LeFrancois and colleagues may be the nature of the experimental model used. For example, as mentioned in Chapter 3, the period of IL-2 production by CD8 cells is thought to be limited due to the manifestation of AINR (Tham *et al.*, 2002). While CTL generated during this period of autocrine IL-2 consumption do not seem to be functionally impaired (Deeths and Mescher, 1997; Harding and Allison, 1993; Azuma *et al.*, 1992) and would be able to control the acute phase of an infection, it is thought that effective CTL function beyond this period requires an exogenous source of IL-2 to control chronic infections. Studies have suggested that CD4 cells may act as the paracrine source of IL-2 for sustained CTL function. Certainly in models of chronic infection, such as viral challenge (Kalams and Walker, 1998; Cardin *et al.*, 1996; Battegay *et al.*, 1994; Matloubian *et al.*, 1994; Kirberg *et al.*, 1993) and tumours (Marzo *et al.*, 2000; Shrikant *et al.*, 1999; Ossendorp *et al.*, 1998), the presence of CD4 cells were clearly

important to a sustained CTL response. However, CD4 cells may also provide indirect assistance to CD8 CTL generation/function through CD40-CD40L interactions that occur between CD4 cells and APC (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). In the LeFrancois paper they used IL-$2^{-/-}$ mice that had CD4 and CD8 cells, thus it is possible that in the absence of IL-2, CD40L interactions via the CD4 cells were able to compensate for the loss of IL-2. In our model, we do not have CD4 cells; therefore CTL generation may be more IL-2 dependent in this circumstance. Certainly, previous work concerning CTL generation in F5 IL-$2^{-/-}$ mice also lacking CD4 cells showed that IL-2 was essential to effector generation but not proliferation (Kramer *et al.*, 1994). Furthermore, IL-15, IL-12, IL-18 and IL-21 have been implicated as having positive effect on CTL generation and function (Zeng *et al.*, 2005; Salcedo *et al.*, 2004; Chen *et al.*, 2000; Okamura *et al.*, 1995; Zou *et al.*, 1995) and research suggests that IL-15 is produced by dendritic cells after CD40-CD40L interactions (Kuniyoshi *et al.*, 1999). In the polyclonal mice used by LeFrancois and colleagues, the presence of CD4 cells may also regulate the expression of these molecules by virtue of an effect on APC function, compensating for the loss of IL-2. Again, in our system, we may not be inducing efficient expression of these cytokines, possibly as an indirect consequence of the lack of CD4 cells to condition the APC. Therefore, in a polyclonal setting, the loss of IL-2 may be less detrimental to CTL generation. However increasing the concentration of IL-2 may still be beneficial in these circumstances as shown by Ahmed and colleagues who gave IL-2 to mice during the contraction phase of LCMV infection and noted that the subsequent frequency of viral-specific CD8 cells was increased compared to

untreated control mice (Blattman *et al.*, 2003). The real importance of IL-2 availability to a CTL response may become evident in situations such as HIV infection where the immune system is unbalanced by a decline in CD4 numbers.

Although the role of IL-2 in CTL generation is dependent upon the experimental model, it is established that, with the exception of Granzyme B, the promoter elements of these genes all contain elements that can bind STAT5, which is itself regulated by IL-2 receptor derived signals (Nakajima *et al.*, 1997). What distinguishes a CTL from a naïve CD8 cell is the expression of effector molecules such as Perforin, Granzyme B and IFNγ, therefore, one could argue that IL-2 must exert some effect on the expression of these molecules via their promoters, and thus influence the quality of CTL generated. Indeed our data supports this view, as we show a direct correlation between IL-2 exposure and effector generation/function in terms of the expression of key molecules both at the protein and mRNA levels.

It has been suggested that there is a linear developmental pathway from naïve CD8 cells to effector then on to memory cells (Kaech *et al.*, 2002). This development has been tracked at the genetic level using microarrays and this identified a number of phenotypic changes that naïve cells undergo during the transition to memory cells (Kaech *et al.*, 2002). Expression of CD44 and CD5 is increased during the transition from a naïve to a memory CD8 cell (Kaech *et al.*, 2002). Furthermore, Kaech et al show that CD44 expression is lower on an effector cell in comparison to a resting memory cell. Therefore it was interesting that the upregulation of CD44 and CD5

was reduced by our F5 Fyn$^{-/-}$ cells compared to F5 WT cells. This suggests subtle differences in the programme of CD8 differentiation in the absence of Fyn that may affect the CTL response.

One would expect that in order for an effector cell to become a memory cell it would have to first enter a resting state ready for reactivation on secondary antigen encounter. Certainly the hallmark of a CD8 memory cells is that they can mount a more vigorous response to antigen rechallenge in comparison to a primary effector cell (Kaech *et al.*, 2002; Pihlgren *et al.*, 1996). In the absence of Fyn, the period of naive CD8 cell activation seemed to be prolonged, possibly due to the inability of the PAG/Csk/PEP complex to downmodulate the signalling and this led to increased IL-2 production by F5 Fyn$^{-/-}$ cells (see Chapters 3 and 4). Work by Manjunath and co-workers has suggested that in situations where IL-2 is absent or present in low concentrations, a proportion of partially differentiated CD8 cells may overlook the effector stage all together and directly become memory cells (Manjunath *et al.*, 2001). Therefore, while IL-2 may be able to prolong the effector state, it may concomitantly have a detrimental effect on the generation of memory cells in the absence of Fyn due to a decreased ability to become quiescent. In F5 WT cells, although not as efficient CTL after primary activation, there may be no such defects in generating memory cells as IL-2 production during the primary response was very low (see Chapter 3).

We have begun to look at CD8 memory generation and the function of these cells in the absence of Fyn by conducting *in vivo* killing assays. In this system, we transferred F5 cells (WT or $Fyn^{-/-}$) with live flu virus into Rag-$1^{-/-}$ hosts. Then 28 days later, we transferred in equal numbers of peptide pulsed target cells mixed 1:1 with unpulsed targets in order to measure the memory response. Both populations were labelled with different concentrations of CFSE to distinguish one from another. Then 18 hrs after transfer, we sacrificed the mice and analysed the cells for disappearance of the peptide pulsed target cell peak. Interestingly by this system we noted that F5 $Fyn^{-/-}$ cells, although potent killers after 3 days *in vitro*, were now less efficient compared to WT F5 memory cells at 28 days following initial transfer (data not shown). Moreover, the frequency of CD8 cells in the blood and cell recoveries in the LN were reduced in mice that received F5 $Fyn^{-/-}$ cells compared to F5 WT cells (data not shown). After 7 days, it is likely that the F5 cells have already become resting memory cells as cell numbers are contracting by this time indicating that the peak of the proliferative phase is now over (data not shown). Upon rechallenge one would expect that these resting cells could mount a memory-like response to the antigen. It is possible that after 28 days of *in vivo* activation, while resting memory cells have been formed from the F5 WT cells activated with virus, the F5 $Fyn^{-/-}$ cells had not done so as efficiently. Certainly, memory cells are able to undergo homeostatic proliferation, whereas effector cells do not (Tuma and Pamer, 2002). This could explain why the numbers of F5 $Fyn^{-/-}$ cells were reduced compared to WT, as the more effector-like cells would be unable to expand and fill the empty space. The best way to resolve the question of memory formation in the absence of Fyn

using the F5 system would be to do a detailed analysis of the kinetics of cell expansion and contraction after *in vivo* flu activation. It may also be informative to try and look at the *in vivo* killing capacity of these cells earlier than 7 days.

Although we have suggested that the modulatory role of Fyn in T cells signalling may be exerted through the PAG/Csk/PEP complex, it is possible that other molecules could be involved. Recently, Fyn has been implicated in the signal transduction of another T cell receptor called SLAM that may have an immunomodulatory role (Cannons *et al.*, 2004; Davidson *et al.*, 2004; Chan *et al.*, 2003). The Fyn mediated signal transduction through SLAM receptors requires the adapter molecule SAP (Latour *et al.*, 2001) and its SH2 domain been shown to associate with the SH3 domain of Fyn (Latour *et al.*, 2003). Interestingly, ligation of SLAM with antibodies was originally thought to mimic physiological SLAM ligation, however studies have shown that it in fact blocks SLAM ligand associations (Mavaddat *et al.*, 2000). Furthermore, constitutive engagement of SLAM by its natural ligand, SLAM itself, led to the abrogation of IFNγ production in a cell line (Latour *et al.*, 2001). This suggested that normal SLAM interactions serve to downregulate IFNγ production by CD8 T cells. Furthermore, $SAP^{-/-}$ mice exhibit elevated IFNγ production upon activation via the TCR (Czar *et al.*, 2001; Wu *et al.*, 2001). Also, ligation of SLAM with the blocking antibody has also been shown to enhance cytotoxicity (Henning *et al.*, 2001). Therefore it is possible that in the absence of Fyn, the increase in IFNγ production and CTL function we observed might be due in part to defects in SLAM signalling that would normally dampen a CTL response. However, there are reasons

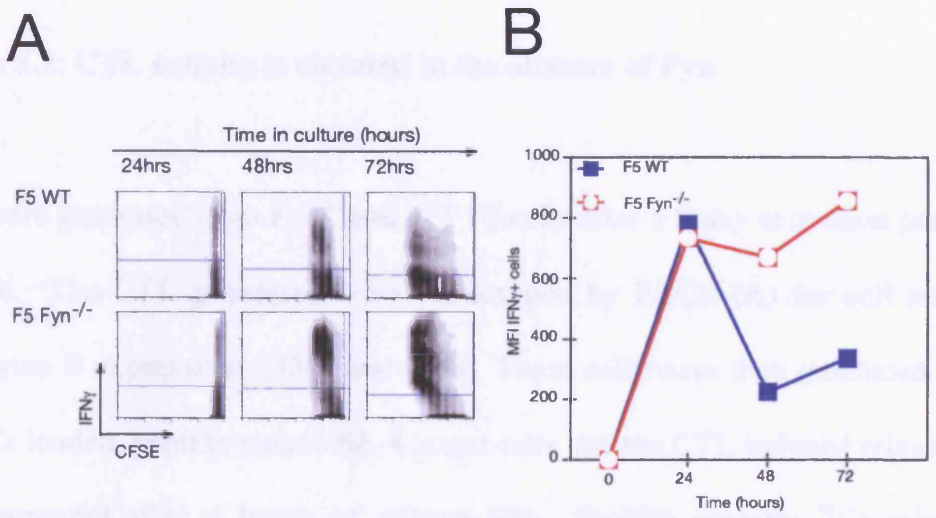
as to why this may not be the case. Firstly, we have shown that the increase in IFNγ production is sensitive to IL-2, in contrast a study by Aversa and colleagues suggested that SLAM mediated effects on T cell activation were IL-2 independent (Aversa *et al.*, 1997). Secondly, we were able to see the maximal effect on IL-2 and IFNγ mRNA production in the absence of Fyn at approximately 4-5 hrs after T cell activation (see Chapter 3 and Figure 5.1) however studies have suggested that SLAM expression is detectable at 6-8 hrs and peaks by 24 hrs (Aversa *et al.*, 1997; Cocks *et al.*, 1995), and that expression of SLAM is also IL-2 independent (Aversa *et al.*, 1997). Therefore SLAM signalling may be too distal an event to directly influence our phenotype, and is clearly not influenced by IL-2 levels. However, our data do not fully rule out that defective SLAM signalling is exerting some effect on the F5 CD8 T cells in a more subtle way. Therefore it may be of interest to look at SLAM mediated signals in our cells.

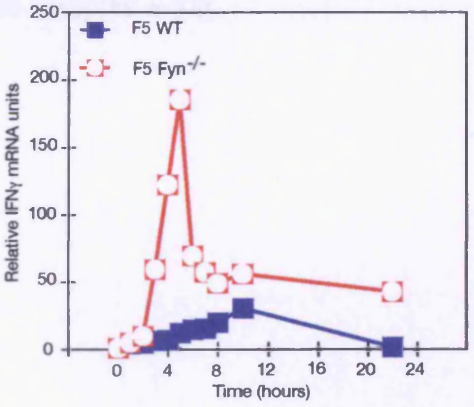
For future experiments, another model that we could exploit to look at the *in vivo* CTL activity of $Fyn^{-/-}$ cells would be to infect mice intranasaly with the A/NT/60-68 strain of influenza virus. This system has been used previously to ask what contribution virus-specific CD8 T cells have to the response against influenza (Moskophidis and Kioussis, 1998). They found that at relatively high doses of virus, the presence of antigen specific CD8 cells in combination with a lack of B cell-derived antibody responses, led to increased morbidity in mice due to vigorous CTL responses and immune pathology. However at lower doses of virus the presence of F5 cells actually had a protective influence. In our situation, we may expect that F5

$Fyn^{-/-}$ mice may show increased morbidity to high viral doses due to an increased CTL response that would cause immune pathology, but an increase in survival to low dose challenge compared to Fyn sufficient animals. This would give a physiological *in vivo* read out of CTL function in the absence of Fyn.

Figure 5.1: $Fyn^{-/-}$ cells produce more IFNγ than WT cells after activation

CFSE labelled cells from $Fyn^{-/-}$ and WT F5 mice (as indicated) were activated using NP68 pMHC. IFNγ production was assessed by intracellular cytokine staining (A and B). Gates were set as shown in A, and the MFI of IFNγ production was plotted against time in culture (B). These data are representative of four independent experiments. The level of IFNγ mRNA was also assessed using real time PCR and values are plotted relative to the housekeeping gene HPRT (C). These data are representative of two independent experiments. Finally, intracellular staining was used to measure IFNγ production by purified naïve CD8 cells from polyclonal $Fyn^{-/-}$ and B6 mice after activation with anti-CD3/CD8 coated micro-beads for 72 hrs (D). The MFI of IFNγ production is shown.


A
B
Time in culture (hours)
24hrs
48hrs
72hrs
F5 WT
F5 Fyn-/-
IFNγ
CFSE
MFI IFNγ+ cells
Time (hours)


C
F5 WT
F5 Fyn-/-
Relative IFNγ mRNA units
Time (hours)

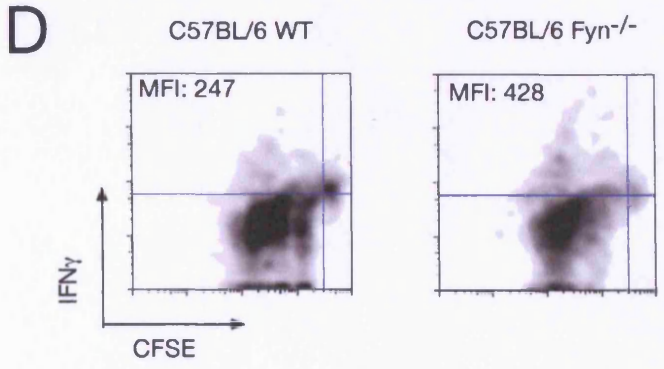
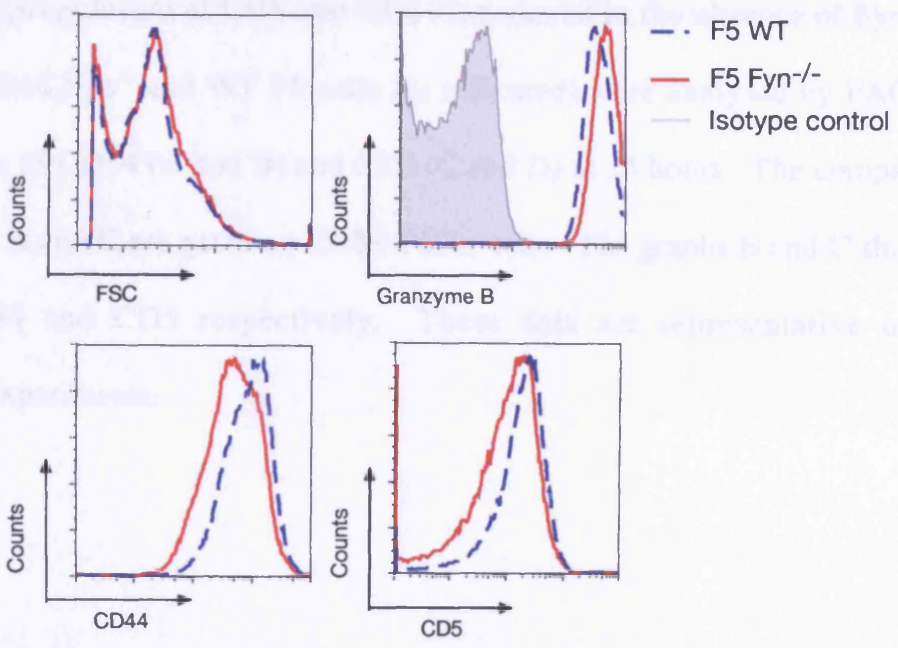

D
C57BL/6 WT
C57BL/6 Fyn-/-
MFI: 247
MFI: 428
IFNγ
CFSE

Figure 5.2: CTL activity is elevated in the absence of Fyn

CTL were generated from $Fyn^{-/-}$ and WT F5 cells after a 3-day activation period with peptide. The CTL generated were phenotyped by FACS (A) for cell size (Fsc), Granzyme B expression, CD44 and CD5. These cells were then incubated with 1 x 10^4 ^{51}Cr loaded, peptide pulsed EL-4 target cells and the CTL induced release ^{51}Cr of was measured after 4 hours of culture (B). Peptide specific ^{51}Cr release was calculated as outlined in the materials and methods, and values plotted are the mean of triplicate samples ± SD.

A


F5 WT
F5 Fyn-/-
Isotype control
Counts
FSC
Counts
Granzyme B
Counts
CD44
Counts
CD5

B

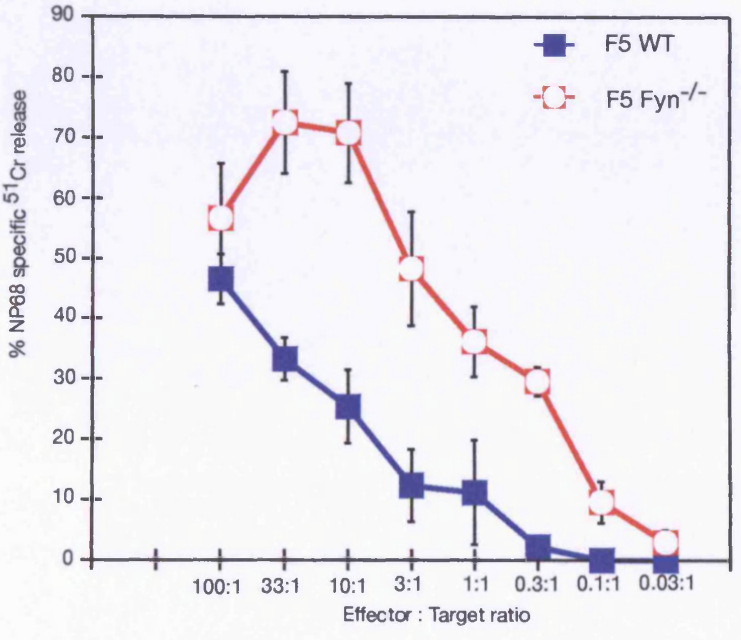
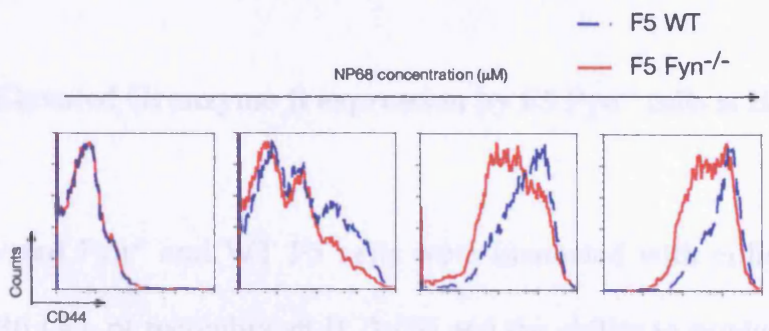

F5 WT
F5 Fyn-/-
% NP68 specific 51Cr release
90
80
70
60
50
40
30
20
10
0
100:1
33:1
10:1
3:1
1:1
0.3:1
0.1:1
0.03:1
Effector : Target ratio

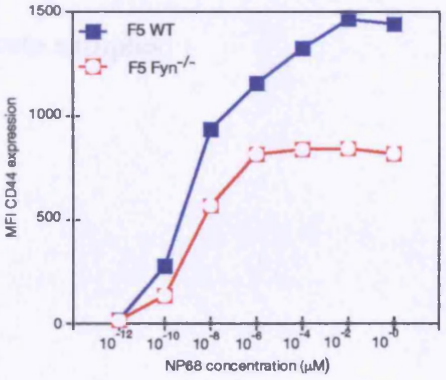
Figure 5.3: Upregulation of CD5 and CD44 is reduced in the absence of Fyn

Peptide activated $Fyn^{-/-}$ and WT F5 cells (as indicated) were analysed by FACS for the expression of CD44 (A and B) and CD5 (C and D) at 24 hours. The comparative histograms in A and C are gated on CD8+ TCR+ cells. The graphs B and C show the MFI of CD44 and CD5 respectively. These data are representative of two independent experiments.

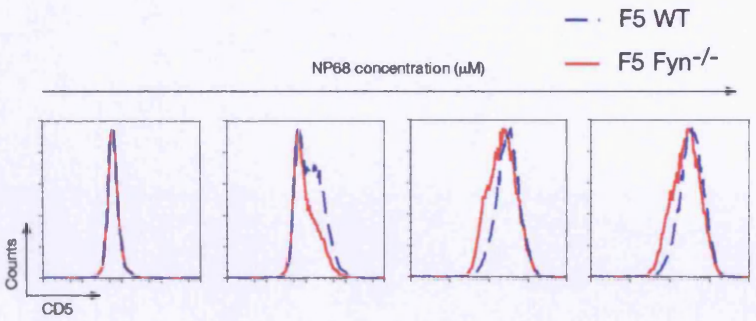
A


F5 WT
F5 Fyn-/-
NP68 concentration (μM)
Counts
CD44

B


F5 WT
F5 Fyn-/-
MFI CD44 expression
1500
1000
500
0
10-12 10-10 10-8 10-6 10-4 10-2 10-0
NP68 concentration (μM)

C


F5 WT
F5 Fyn-/-
NP68 concentration (μM)
Counts
CD5

D

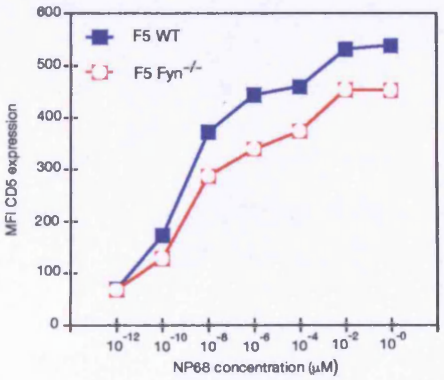
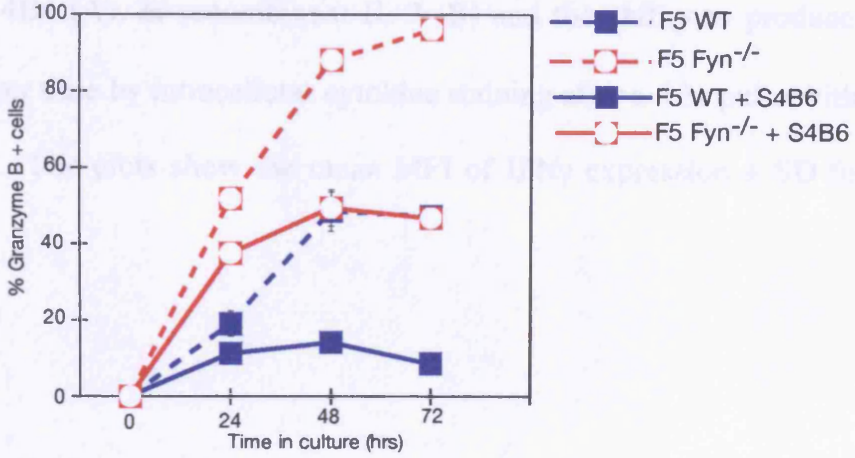

F5 WT
F5 Fyn-/-
MFI CD5 expression
600
500
400
300
200
100
0
10-12 10-10 10-8 10-6 10-4 10-2 10-0
NP68 concentration (μM)

Figure 5.4: Elevated Granzyme B expression by F5 Fyn$^{-/-}$ cells is IL-2 dependent

Peptide activated Fyn$^{-/-}$ and WT F5 cells were incubated with either the anti-IL-2 antibody S4B6 (A), or recombinant IL-2 (B) and the ability to produce Granzyme B was assessed over time. The plots show the mean MFI of Granzyme B expression ± SD for triplicate samples.

A


100
80
60
40
20
0
% Granzyme B + cells
0
24
48
72
Time in culture (hrs)
F5 WT
F5 Fyn-/-
F5 WT + S4B6
F5 Fyn-/- + S4B6

B

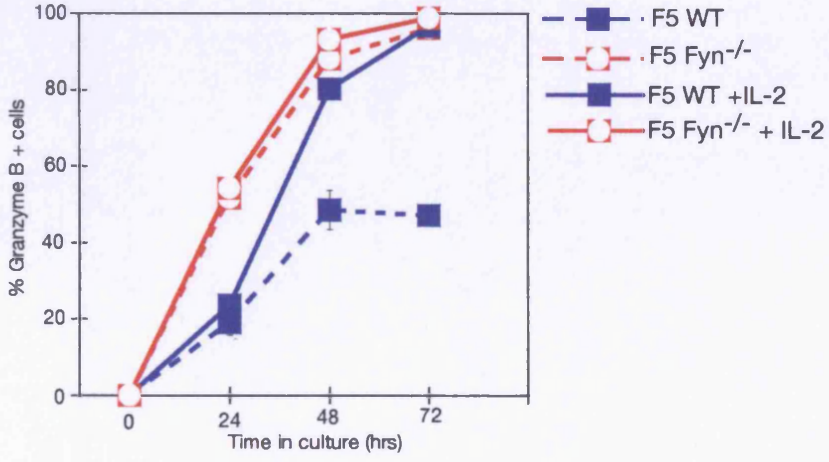
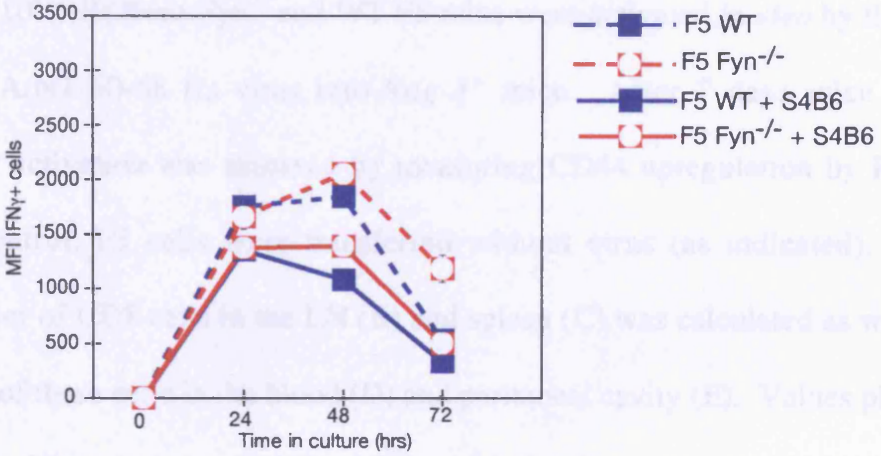

100
80
60
40
20
0
% Granzyme B + cells
0
24
48
72
Time in culture (hrs)
F5 WT
F5 Fyn-/-
F5 WT +IL-2
F5 Fyn-/- + IL-2

Figure 5.5: Elevated IFNγ expression by F5 Fyn$^{-/-}$ cells is also IL-2 dependent

Peptide activated Fyn$^{-/-}$ and WT F5 cells were incubated with either the anti-IL-2 antibody S4B6 (A), or recombinant IL-2 (B) and the ability to produce IFNγ was assessed over time by intracellular cytokine staining after a 4 hr pulse with PdbU and ionomycin. The plots show the mean MFI of IFNγ expression ± SD for triplicate samples.

A


3500
3000
2500
2000
1500
1000
500
0
MFI IFNγ+ cells
0
24
48
72
Time in culture (hrs)
F5 WT
F5 Fyn-/-
F5 WT + S4B6
F5 Fyn-/- + S4B6

B

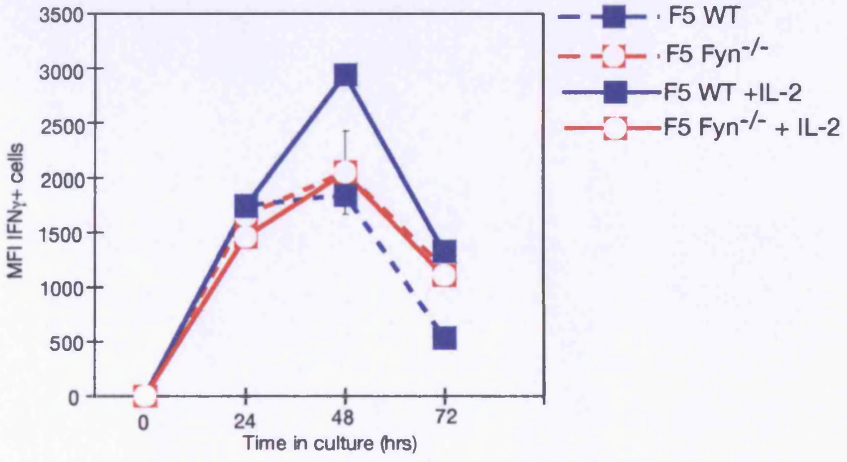
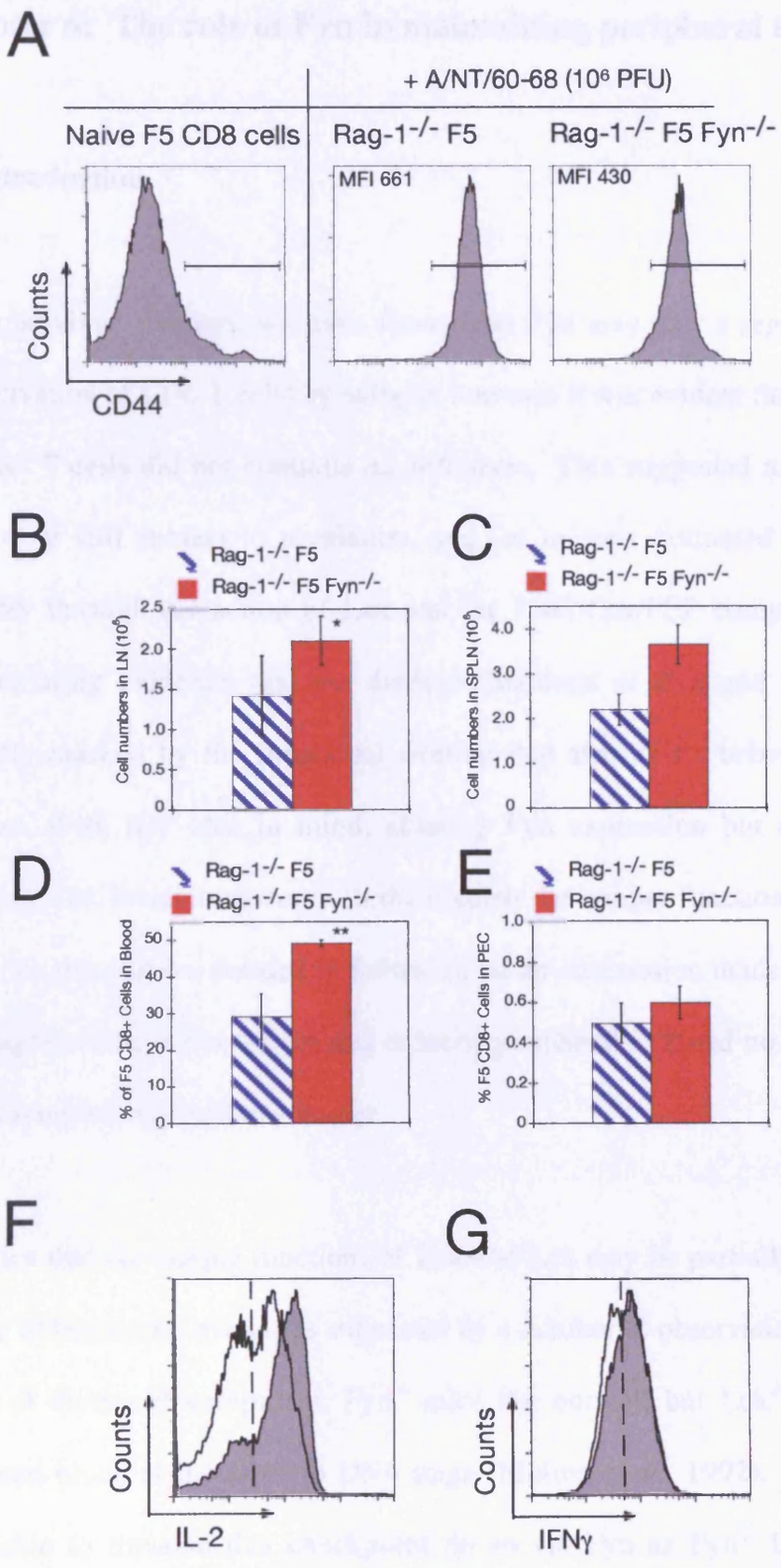

3500
3000
2500
2000
1500
1000
500
0
MFI IFNγ+ cells
0
24
48
72
Time in culture (hrs)
F5 WT
F5 Fyn-/-
F5 WT +IL-2
F5 Fyn-/- + IL-2

Figure 5.6: Activation of F5 Fyn$^{-/-}$ cells *in vivo* leads to elevated IL-2 and IFNγ production and increased cell recoveries.

A total of 3 x 10^6 cells from Fyn$^{-/-}$ and WT F5 mice were activated *in vivo* by the co-transfer with A/NT/60-68 flu virus into *Rag-1*$^{-/-}$ mice. After 7 days mice were sacrificed and activation was assessed by measuring CD44 upregulation by FACS (A). As a control, F5 cells were transferred without virus (as indicated). The absolute number of CD8 cells in the LN (B) and spleen (C) was calculated as well as the frequency of these cells in the blood (D) and peritoneal cavity (E). Values plotted are the mean ± SD, where n = 5. The ability of LN cells to produce IL-2 (F) and IFNγ (G) after recall stimulation and intracellular cytokine staining was determined. These data are representative of 3 independent mice. Statistical significance was determined using the t-test where * = $p<0.05$ and ** = $p<0.01$.


A
+ A/NT/60-68 (10⁶ PFU)
Naive F5 CD8 cells
Rag-1-/- F5
Rag-1-/- F5 Fyn-/-
MFI 661
MFI 430
Counts
CD44
B
Rag-1-/- F5
Rag-1-/- F5 Fyn-/-
Cell numbers in LN (10⁶)
C
Cell numbers in SPLN (10⁶)
D
% of F5 CD8+ Cells in Blood
E
% F5 CD8+ Cells in PEC
F
Counts
IL-2
G
IFNγ

Chapter 6: The role of Fyn in maintaining peripheral tolerance

6.1 Introduction

In the previous chapters, we have shown that Fyn may play a regulatory role in the activation of CD8 T cells by antigen, however it was evident that the response of $Fyn^{-/-}$ T cells did not continue *ad infinitum*. This suggested to us that $Fyn^{-/-}$ cells were still subject to regulation, and for reasons discussed in Chapter 4, possibly through the action of Lck and the PAG/Csk/PEP complex. There is accumulating evidence that the distinct functions of Fyn and Lck could be partially masked by the functional overlap that also exists between these two kinases. With this idea in mind, ablating Fyn expression but concomitantly reducing Lck levels may serve to distinguish the unique functions of Fyn in T cells. To this end we decided to follow up on an observation made in the lab that a transgenic mouse line expressing reduced peripheral Lck and no Fyn presented with a lymphoproliferative disorder.

The idea that the unique functions of Fyn and Lck may be partially masked by a degree of functional overlap is supported by a number of observations. Firstly, in terms of thymic development, $Fyn^{-/-}$ mice are normal, but $Lck^{-/-}$ mice have a profound block at the DN3 to DN4 stage (Molina *et al.*, 1992). However, the cells able to traverse this checkpoint do so via Fyn as $Fyn^{-/-}$ $Lck^{-/-}$ mice are completely arrested at this stage (Groves *et al.*, 1996; van Oers *et al.*, 1996). Moreover, elevated Fyn expression has been shown to be able to rescue thymic development in $Lck^{-/-}$ mice (Groves *et al.*, 1996). These studies suggest that one of

the preferential functions of Lck is to transmit the signals required for β selection and that WT levels of Fyn can also do this but with a much lower efficiency that can be improved if Fyn is over expressed. Secondly, in terms of the *in vitro* activation of peripheral T cells, we have found in our lab that while Lck deficiency has a detrimental effect on the triggering of both CD4 (Lovatt et al., manuscript submitted) and CD8 cells (data not shown) into division, Fyn can compensate as proliferation is still be observed, albeit at a higher concentration of anti-TCR stimuli. Again, this is Fyn specific, as Lck^{Ind} $Fyn^{-/-}$ cells in which Lck expression is turned off by the removal of Dox cannot be activated in response to TCR stimulation (unpublished observations). These data suggest that the preferential function of Lck in peripheral T cells is to promote activation, which Fyn may also do so but with reduced efficiency. Finally, in terms of *in vivo* peripheral function, our lab has shown that both Lck and Fyn can transmit TCR signals required for T cell survival (Seddon and Zamoyska, 2002b), but that Lck is required for driving TCR-mediated homeostatic proliferation as Fyn was unable to compensate in the absence of Lck (Seddon and Zamoyska, 2002a). Again these data suggest that the function of these two kinases has overlap, but that they may have preferential roles in T cell responses. Therefore, Lck seems more able than Fyn to drive a proliferative signal, but judging from our data, Fyn may be more required to efficiently downmodulate a response.

At the molecular level, there is also evidence for both distinct and overlapping functions. For example Fyn and Lck have been shown to interact with intracellular molecules such as Zap-70 (Fusaki *et al.*, 1996; Duplay *et al.*, 1994), PI-3 kinase (Susa *et al.*, 1996), c-Cbl (Hawash *et al.*, 2002; Tsygankov *et al.*,

1996), and LIME (Brdickova *et al.*, 2003; Hur *et al.*, 2003). However, studies have also shown that there may be distinct substrates, as Fyn, but not Lck, has been shown to associate with ADAP (Marie-Cardine *et al.*, 1999), SAM68 (Feuillet *et al.*, 2002) and SAP (Chan *et al.*, 2003). In contrast work has suggested Lck uniquely associates with molecules such as LckBP1 (Takemoto *et al.*, 1995), and LAD (Choi *et al.*, 1999). While the functional outcome of these interactions remain to be fully elucidated, again it suggests that both overlapping and distinctive function exist between Lck and Fyn.

As mentioned previously, T cell development is grossly defective in $Lck^{-/-}$ mice (Molina *et al.*, 1992), and completely blocked in $Lck^{-/-}$ $Fyn^{-/-}$ mice (Groves *et al.*, 1996; van Oers *et al.*, 1996), therefore we have developed a system in which Lck expression can be regulated at the transcriptional level (Lck^{Ind} mice) (Legname *et al.*, 2000). This was achieved using the tetracycline-responsive gene induction system (see Figure 6.1A) (Gossen *et al.*, 1995; Furth *et al.*, 1994). When the tetracycline derivative Doxycycline (Dox) is present the constitutively expressed "tet-on" transactivator domain (rtTA) under the control of the huCD2 expression casette (Zhumabekov *et al.*, 1995) binds to the tetracycline response element upstream of the minimal CMV promoter. This drives expression of the downstream Lck transgene that can compensate for the absence of endogenous Lck protein in $Lck^{-/-}$ mice. Using this system we are able to generate a peripheral T cell population comparable to a WT mouse by administering Dox to pregnant mothers and also during weaning (Legname *et al.*, 2000). We can then remove Dox from the diet of the offspring at any given time in order to analyse peripheral T cell function in the absence of this kinase and have shown that all detectable

Lck is gone by 7 days (Seddon *et al.*, 2000). However, one outcome of this system is that, while levels of Lck in transgenic mice are comparable to Lck in a WT thymus, in peripheral T cells expression from the Lck^{Ind} system is ~20% of WT levels (see Figure 6.1B)(Seddon *et al.*, 2000). While the reason for this is unclear, studies from our lab have shown that T cells generated using this system behave similarly to T cells from a Lck WT mouse in terms of thymic development and peripheral function (Seddon and Zamoyska, 2002a; Seddon and Zamoyska, 2002b; Legname *et al.*, 2000; Seddon *et al.*, 2000). When we crossed the Lck^{Ind} mice onto a $Fyn^{-/-}$ background, again thymic development proceeded normally, however there was a reduction in peripheral T cell numbers compared to Fyn sufficient controls (Seddon and Zamoyska, 2002b). Interestingly, we observed that the continual administration of Dox diet caused the animals to lose weight, necessitating the culling of the mice. This did not seem to be caused by the Dox diet *per se*, as B6 mice receiving this supplement remained healthy (see Figure 6.1C). In this chapter I will present the basic characterisation of this phenotype. For clarity, I will refer to the $Lck^{-/-}$ mice with the rtTA inducible Lck transgene as Lck^{Ind} mice, and those also lacking Fyn expression as Lck^{Ind} $Fyn^{-/-}$ mice.

6.2 Mice expressing reduced Lck and no Fyn present with wasting disease and inflammation of the upper GI tract

To confirm that the manifestation of the wasting disease was specific to reduced Lck levels and ablated Fyn expression, and also to determine the time-scale of appearance, we monitored the weights of mice expressing all possible combinations of Fyn and Lck. From the data presented in Figure 6.2A it was

clear that Dox fed Lck^{Ind} $Fyn^{-/-}$ mice were the only group that exhibited weight loss over time. Moreover, if mice with the same genotype were not fed Dox food, and thus did not express either Lck or Fyn, then no weight loss was observed. The disease was not caused by administration of Dox alone as B6 mice that were fed this diet remained healthy. Furthermore Dox fed Lck^{Ind} mice that expressed Fyn also showed no sign of weight loss, indicating that Fyn expression was protective. When the weight of the mice presenting with wasting disease dropped to ~85% of the starting values they were euthanised for further analysis. As Figure 6.2A shows, this occurred between 8 and 24 weeks of age, with no sign of disease before 4 weeks of age.

We wanted to determine what could be the cause of the wasting disease, therefore mice were monitored daily for signs of diarrhoea or appetite loss. Mice from all groups fed comparably and showed no signs of watery stools in the cages. We then proceeded to remove the entire gastrointestinal (GI) tracts from sick animals for analysis. Figure 6.2B shows photos taken of the upper regions of the gut from a B6 mouse (left panel) and a Dox fed Lck^{Ind} $Fyn^{-/-}$ mouse presenting with weight loss (right panel). As shown in this example, the GI tract from sick mice showed regions of swelling marked on the photo with white arrows. In contrast these regions of swelling and thickening were not observed in the GI tract from the B6. The thickening and swelling was restricted to the upper section and not in the colons of sick mice (data not shown), thus making the disease phenotypically distinct from other models of IBD that involve the colon (Leithauser *et al.*, 2002).

When sections were taken through the swollen areas of the upper GI tract from sick mice (Figure 6.2C), H & E staining revealed the presence of cellular infiltrate in the villi (marked with arrows). Furthermore swelling and the presence of cell infiltrate was not observed in the gut from Dox fed B6 mice, Lck^{Ind} $Fyn^{-/-}$ animals not presenting with weight loss and any of the other mice listed in Figure 6.2A (data not shown).

Figure 6.3 Dox fed Lck^{Ind} $Fyn^{-/-}$ mice exhibit enlarged LN and elevated T cell numbers.

As well as a wasting disease, further analysis of the sick Dox fed Lck^{Ind} $Fyn^{-/-}$ mice revealed that they possessed enlarged lymph nodes compared to control mice (Figure 6.3A). Prior to disease manifestation (~ 4 weeks of age), the frequency (Figure 6.3B) and absolute number (Figure 6.3C) of T cells in the peripheral lymph nodes of Dox fed Lck^{Ind} $Fyn^{-/-}$ mice was 2-fold less than B6 control mice. However, when disease was observed, the frequency of T cells increased to that of the B6 LN. This increase in frequency did not always translate into an increase in absolute T cell numbers, but in a proportion of the animals, numbers of T cells were substantially increased compared to B6 controls. It should also be mentioned that in some mice B cell numbers also increased.

As the mice presented with GI tract pathology, the mesenteric lymph nodes (MLN) were analysed separately. Again, Figure 6.3D shows that the frequency of T cells in the MLN of healthy Lck^{Ind} $Fyn^{-/-}$ mice (4 weeks old) was ~2.5 fold less than the percentage in a B6 mouse. However, with age and once disease was

manifested, the frequency of T cells increased compared to the frequency in the B6 mouse. Figure 6.3E shows that while the absolute T cell number in the MLN of B6 mice remained constant with age, in the sick mice there was a clear increase in cell number over time. A greater percentage of Lck^{Ind} $Fyn^{-/-}$ mice exhibited T cell lymphoproliferation in the MLN than in the peripheral lymph nodes (PLN), indicating that the T cell proliferation was more profound at this site.

6.4 The expansion of CD4 cells is the main reason for the increase in T cells

We wanted to determine which T cell subset(s) was responsible for the increase in overall T cell numbers observed in sick mice. To this end we carried out extensive phenotyping of Dox fed Lck^{Ind} $Fyn^{-/-}$ mice presenting with disease. Figure 6.4A shows that before showing signs of sickness the frequency of CD4 cells and CD8 cells was reduced by 2-fold and 7-fold respectively in the PLN compared to a B6 mouse. When presenting with disease the frequency of CD4 cells increased 2-fold and, in the example shown in Figure 6.4A, the frequency of CD8 cells increased ~5-fold. In the FACS plots shown in Figure 6.4A and D the differences in the level of CD8 expression between healthy and sick Lck^{Ind} $Fyn^{-/-}$ mice reflect the fact that staining was carried out on different days, to highlight this, age matched B6 stains are shown. When these frequencies were converted into total cell numbers (Figure 6.4B), CD4 cells increased with age in ~60% of Lck^{Ind} $Fyn^{-/-}$ mice after 9 weeks of age. In contrast, Figure 6.4C shows that expansion of CD8 cell numbers with age was restricted to only ~20% of animals. CD4 and CD8 T cell numbers remained constant in B6 mice with age. It should

also be noted that we only observed an increase in CD8 numbers when CD4 numbers were also elevated.

As before, the MLN were analysed separately from the PLN. Figure 6.4D shows that a similar frequency of CD4 and CD8 T cells existed in the MLN of healthy Lck^{Ind} $Fyn^{-/-}$ mice compared to PLN. Again, upon disease manifestation, the frequencies of CD4 and CD8 cells increased, however the expansion of CD4 cells in the MLN was 3-fold compared to only 2-fold in the PLN. Furthermore, CD8 expansion, although evident, was also not as great as was observed in other the lymph nodes. When the absolute numbers of CD4 cells (Figure 6.4E) and CD8 cells (Figure 6.4F) were plotted with age it revealed that expansion of CD4 numbers again occurred over time, and was evident in nearly every mouse analysed over 4 weeks of age. In contrast only 3 mice out of 17 showed increased CD8 cell numbers in the MLN. Collectively these data indicated that CD4 cells were the main population responsible for the increase in T cell numbers and the major lymphoid site where this occurred was in the mesenteric lymph nodes.

6.5 CD4 cell expansion occurs within the memory compartment

Having identified expansion of CD4 cell numbers as the major contributor to the increase in T cell numbers observed in sick mice, we wanted to determine the phenotype of these cells. CD4 cells can be subdivided into memory and naïve based on the expression of the marker CD44 (Dutton *et al.*, 1998). Upon activation by antigen, or in response to lymphopenia, a CD4 cell will upregulate CD44 and this is considered to be a marker of antigen-experienced cells (Zola,

2000). To this end we looked at the frequency of CD4+ CD44+ cells within the PLN from healthy mice and compared these values to mice presenting with disease as well as with B6 animals. Figure 6.5A shows that before disease, CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice showed a reduced frequency of $CD44^{hi}$ CD4 cells compared to a B6 control mouse. However, upon disease manifestation, the frequency of $CD44^{hi}$ cells increased ~3-fold compared to that seen in a B6 mouse. When the absolute number of naive $CD44^{lo}$ (Figure 6.5B) and $CD44^{hi}$ memory (Figure 6.5C) CD4 cells were plotted against the age of the mouse it was evident that there was an expansion of CD4 memory cell numbers with age in the PLN of sick mice. In contrast naïve CD4 cell numbers remained comparable to B6 controls.

When the same analysis of the CD4 cells from the MLN was carried out, a similar pattern emerged to that observed in the PLN. Figure 6.5D shows that there was a reduced frequency of $CD44^{hi}$ CD4 cells in the MLN of healthy Lck^{Ind} $Fyn^{-/-}$ mice compared to B6 controls. As the mice became sick the frequency of $CD44^{hi}$ CD4 cells increased 3-fold compared to B6 controls. When the absolute number of naïve (Figure 6.5E) and memory (Figure 6.5F) CD4 cells was plotted against mouse age, again there was a striking expansion in the memory CD4 T cell pool over time within the MLN, also with no change in naïve CD4 numbers. As was described previously for both total T cell numbers and CD4 cell numbers a greater frequency of sick Lck^{Ind} $Fyn^{-/-}$ mice exhibited an elevation of memory CD4 cells in the MLN compared to other peripheral lymph node sites.

6.6 CD4 cells from sick mice have an activated phenotype and remain polyclonal

Disregulation of Src family kinases has been shown to lead to T cell transformation (Majolini *et al.*, 1999; Abraham *et al.*, 1991), therefore we wanted to investigate if CD4 cell expansion in our sick mice was a result of the outgrowth of a specific T cell clone(s). To this end we used a panel of anti-TCR Vβ chain antibodies and determined the frequency of CD4 cells expressing specific β chains by FACS. Figure 6.6A shows the percentage of CD4 cells that expressed the indicated Vβ chains from groups of B6 mice, 4 week old healthy Lck^{Ind} $Fyn^{-/-}$ mice, and mice showing signs of disease. It is clear that similar frequencies of CD4 cells expressing the panel of Vβ chains were observed in all mouse groups. There were however some subtle differences between the three groups of mice. For example healthy Lck^{Ind} $Fyn^{-/-}$ mice had a higher percentage of CD4 $V\beta5^{+}$ and CD4 $V\beta6^{+}$ cells compared to both sick Lck^{Ind} $Fyn^{-/-}$ and B6 control mice. Furthermore, no CD4 $V\beta11^{+}$ cells could be detected in the healthy Lck^{Ind} $Fyn^{-/-}$ mice, whereas sick mice contained this population at a very small frequency. Collectively these data showed that CD4 T cell expansion was not due to the outgrowth of any of the Vβ expressing clones tested, but that there may have been subtle changes in the Vβ repertoire between groups of mice.

We next sought to further phenotype the CD4 cells from sick Lck^{Ind} $Fyn^{-/-}$ mice. One hallmark of activated T cells is the ability to make cytokines upon *ex vivo* restimulation (Andersson *et al.*, 1988). Furthermore the ability of these cells to produce cytokine could, in theory, contribute to disease pathogenesis. To this end

we decided to look at the ability of CD4 T cells from Lck^{Ind} $Fyn^{-/-}$ mice to produce IL-2 and IFNγ upon *ex vivo* restimulation with PdbU and ionomycin. Figure 6.6B shows that the percentage of $CD44^{hi}$ CD4 cells isolated from the PLN and MLN able to make IL-2 was increased between 2 to 4-fold respectively compared to the same population from B6 mice. This was restricted to the $CD44^{hi}$ population in both groups as no IL-2 production was detected in the $CD44^{lo}$ CD4 cells. When we addressed IFNγ production (Figure 6.6C) the differences were even more striking. CD4 cells from the PLN and MLN of B6 mice made little IFNγ upon restimulation. In contrast ~14% of CD4 $CD44^{hi}$ cells from the PLN and MLN of sick mice were able to produce IFNγ. Again, no cytokine was detected in the naïve CD4 population. Interestingly CD4 cells from healthy Lck^{Ind} $Fyn^{-/-}$ mice did not shown an increased propensity to make IFNγ (data not shown).

To further elucidate if the CD4 cells from the LN of sick mice had an activated phenotype we looked at the expression of CD45RB and CD62L. Expression of CD62L is high on the surface of naïve CD4 cells, but is shed upon activation (Chen *et al.*, 1995; Tedder *et al.*, 1995b; Tedder *et al.*, 1995a). Furthermore, CD45RB expression is also high on naïve T cells and low on activated cells (Dutton *et al.*, 1998). Analysis of the expression of these markers revealed that CD4 cells from sick mice were predominantly $CD62L^{lo}$, but could be equally subdivided into $CD45RB^{hi}$ or $CD45RB^{lo}$ (Figure 6.6D). In contrast the majority of CD4 cells from B6 mice were $CD62L^{hi}$ and $CD45RB^{hi}$, the same pattern was observed in healthy ≤4-week-old Lck^{Ind} $Fyn^{-/-}$ mice (data not shown).

We also decided to look at the expression of CD5 on the surface of the CD4 cells, as CD5 has been shown to correlate with Src kinase levels (Seddon and Zamoyska, 2002b), and is thought to act as a negative regulator of T cell activation (Tarakhovsky *et al.*, 1995). Figure 6.6E shows that the MFI of CD5 expression was reduced 2-fold on the surface of CD4 cells from sick mice compared to B6 controls, however this did seem to reflect the fact that there were populations of $CD5^{hi}$ and $CD5^{lo}$ cells from the sick Lck^{Ind} $Fyn^{-/-}$ mice. Furthermore, this reduction in CD5 MFI correlated with disease as healthy mice had comparable levels to a B6 and did not have two populations basewd on CD5 expression (data not shown).

6.7 Sick mice have elevated numbers of CD4 $CD25^{+}$ cells

The fact that we observed a general lymphoproliferation of activated CD4 T cells combined with GI tract pathology suggested to us that there could be defects in mechanisms that control peripheral tolerance. Deletion of autoreactive T cells by negative selection relies on the presence of self-pMHC complex within the thymus. While self-antigens from sites such as the GI tract will be expressed in the thymus due to the function of the AIRE transcription factcor (Anderson *et al.*, 2002), T cell clones reactive to peptides produced by commensal gut bacteria will escape deletion as thesesantigens will not be presented in the thymus. To counteract this problem, there are mechanisms that regulate activation of these cells upon encountering self-antigen in peripheral sites. T regulatory cells (T_{reg}) cells mediate one such mechanism. It is thought that these cells work by suppressing the activation of autoreactive T cells (Strober *et al.*, 2002), by

mechanisms yet to be fully elucidated. To this end we wanted to check that these cells were indeed present in the PLN and MLN of mice before and after presenting with sickness. Phenotypically, a major population of T_{reg} cells are generally regarded to be CD4 $CD25^+$, furthermore, the expression of CD4 on the surface of these $CD25^+$ cells is marginally lower than on $CD25^-$ CD4 cells (Sakaguchi *et al.*, 1995). Figure 6.7A and C show the frequency of these putative T_{reg} cells in the PLN and MLN respectively in all groups of mice. It should be noted that the expression of CD4 on the $CD25^+$ cells in all groups was marginally reduced compared to $CD25^-$ cells. Also the frequency of these cells in the B6 mouse was the same regardless of the peripheral location. Furthermore, the absolute number of these putative T_{reg} cells in control mice remained unchanged in the PLN (Figure 6.7B) and MLN (Figure 6.7D) with age. Before disease onset, the frequency of these CD4 $CD25^+$ cells from Lck^{Ind} $Fyn^{-/-}$ mice was comparable to B6 mice, as were the absolute numbers (Figures 6.7B and D). However when sickness presented the frequency of these cells increased dramatically in both the PLN and MLN of sick mice. Furthermore, the absolute numbers in the PLN (Figure 6.8B) and MLN (Figure 6.8D) increased with age and disease incidence. The percentage of sick mice showing elevated numbers of these cells in the MLN was higher than in the PLN (~50% in PLN, ~95% in MLN). Consistent with the memory CD4 expansion in the MLN, the expansion of CD4 $CD25^+$ in the same site could indicate a response to bacterially derived antigens in the GI tract.

6.8 Sick mice have elevated B cell numbers and ANA with age.

Although CD4 cell numbers were clearly elevated in the lymph nodes of sick mice, they did not account for the full increase in cellularity observed. Fyn and Lck are expressed in B cells and there is evidence that certain subsets may have a functional requirement for Lck (Dal Porto *et al.*, 2004). Although we are unsure if the inducible Lck system is expressed in B cells it is a possibility. Furthermore T cell disregulation could influence B cell function possibly through CD40-CD40L interaction that regulate isotype switching (Bishop and Hostager, 2003). Therefore we wanted to assess whether B cell numbers were affected in sick mice. Figure 6.8A, B and C shows the absolute numbers of B cells in the PLN, MLN and spleen respectively from sick mice and B6 controls. It is clear that there is B cell expansion in the PLN and MLN of Lck^{Ind} $Fyn^{-/-}$ mice with age however this trend is less pronounced in the PLN compared to the MLN. As the spleen is a major B cell site we also looked at the B cell numbers there. Figure 6.8C shows that, although more variation within groups was observed, there was also some increase in B cell numbers in the spleens of sick Lck^{Ind} $Fyn^{-/-}$ mice.

Next we decided to address the production of antibodies by B cells by measuring serum Ig isotype levels by ELISA to see if there were any obvious differences. Figure 6.8D shows that in the serum of all mice analysed, levels of IgGa, IgG2b, IgG1, IgG3 and IgM were comparable between B6, and healthy or sick Lck^{Ind} $Fyn^{-/-}$ mice. There was an increase in the level of IgG2a in the serum of Lck^{Ind} $Fyn^{-/-}$ mice compared to B6 controls, however this did not correlate with disease incidence as it was also elevated in healthy ≤ 4-week-old animals. This does not

rule out the possibility that elevation of IgG2a is a very early indicator of disease induction. We also looked at levels of IgE in the serum of mice. Figure 6.8E shows that there was an increase in IgE levels in the serum of sick mice with age. The one animal that exhibited very high IgE levels also presented with irritated and swollen ears in combination with skin lesions (data not shown).

As IgE levels have also been shown to correlate with autoimmune disorders such as Systemic lupus erythematosus (SLE) in humans (Nagpal *et al.*, 1990) and also are detectable the lupus-like flaky skin mutant autoimmune mice (Withington *et al.*, 2002), we decided to look for the presence of anti-nuclear antibodies (ANA). The presence of ANA is often indicative of autoimmunity as it reflects the presence of self reactive antibodies generated as a result of T cell activation (Shlomchik *et al.*, 1990). From Figure 6.8F it is clear that sera from B6 mice did not contain detectable levels of ANA as values were not greater than those obtained with negative control sera. Furthermore, sera from 4 week-old Lck^{Ind} $Fyn^{-/-}$ mice also contained no detectable ANA, but with age and disease progression we were able to detect ANA in the sera of sick Lck^{Ind} $Fyn^{-/-}$ mice.

6.9 Disease manifestation and duration can be controlled by switching Lck expression on or off

Having shown that the disease only became apparent in Dox fed Lck^{Ind} $Fyn^{-/-}$ mice after 4 weeks and becoming fatal between 8 and 24 weeks of age we wanted to address the role that continual Lck expression had in influencing disease progression. To this end litters of Lck^{Ind} $Fyn^{-/-}$ mice were fed Dox up to 4 weeks

of age to generate peripheral T cells. At 4 weeks these mice were taken off Dox and were left for a further 6 weeks during which no sign of disease was noted, as shown previously in Figure 6.2A. The mice were weighed at 10 weeks of age and put back on a Dox diet. Figure 6.9A shows that within 7 days of returning to Dox food and switching Lck expression back on the mice lost 15-20% of their original body weight and had to be culled. The littermate control that did not receive Dox remained healthy. Moreover, when CD4 T cell expansion was measured in the blood of these animals, the frequency of $CD4^+$ PBL increased over time in the Dox fed mice, but not in the mouse kept free from Dox thus not expressing Lck (Figure 6.9B). These data indicated that the Lck^{Ind} $Fyn^{-/-}$ mice required Lck expression for initiating the sickness.

Once the disease had manifested in the mice, we wanted to determine if pathogenesis required the continual expression of Lck. To this end a litter of 6 Lck^{Ind} $Fyn^{-/-}$ mice were fed Dox and the weights were monitored weekly. When an animal's body weight dropped to 90% of the original value we removed Dox and continued to monitor the body mass. Figure 6.9C shows that 5 out of the 6 mice began to show signs of disease around 7 weeks of age. When these animals were removed from Dox food having lost 10% of their original body weight, 3 animals recovered or stabilised their weight loss, and 2 mice which continued to lose weight had to be culled. Examination of the GI tract from the culled mice revealed extensive swelling of the upper regions (data not shown), but there was no sign of increased T cell numbers in the PLN or MLN. In contrast, the GI tract from mice that recovered body weight after being removed from Dox did not show the same extent of swelling and tissue damage (data not shown).

Collectively these data show that disease progression, specifically memory CD4 T cell expansion, required continual Lck expression.

6.10 Transfer of Lck^{Ind} $Fyn^{-/-}$ LN cells can induce wasting disease in Rag-$1^{-/-}$ recipient mice.

Having characterised the nature of the disease observed in Dox fed Lck^{Ind} $Fyn^{-/-}$ mice we wanted to try and begin to understand what could be driving its manifestation. Analysing the disease in theses mice had a number of anomalies that needed to be resolved. For example, we were comparing the phenotype of activated cells from the Lck^{Ind} $Fyn^{-/-}$ mice with cells from B6 animals that were largely naïve. Secondly, we did not know whether the disease was in fact being driven by the activation of auto-reactive T cells or whether it was being driven inpart by activation in response to lymphopenia. This could be significant as healthy Lck^{Ind} $Fyn^{-/-}$ mice have a 2-fold fewer T cells compared to B6 mice (see Figure 6.3). Thirdly, although Dox fed Lck^{Ind} mice expressing Fyn showed no signs of disease (see Figure 6.2A) these mice were reared on a diet containing 1 mg/g of Dox food compared to Lck^{Ind} $Fyn^{-/-}$ mice that received a 3 mg/g diet. The reason for this difference was that we observed poor thymic development in the Lck^{Ind} $Fyn^{-/-}$ mice that had been fed 1 mg/g Dox diet (data not shown). Therefore the higher dose of Dox may have been contributing in some way to the disease. To try and answer all of these discrepencies we decided to use an *in vivo* transfer model. This involved the transfer total cells from the LN of healthy 4 week old Lck^{Ind} $Fyn^{-/-}$ mice and from Lck^{Ind} mice expressing Fyn into separate groups of Rag-$1^{-/-}$ hosts. Furthermore, using this model we could also determine if the

disease was cell intrinsic and could be transferred to recipient mice, or whether the peripheral environment of the Lck^{Ind} $Fyn^{-/-}$ mice was also a contributing factor. Furthermore, we used Lck^{Ind} mice expressing Fyn as controls, which would confirm that Fyn deficiency was essential to the manifestation of the disease.

Before injection, the total cell numbers were adjusted so that equal numbers of T cells (1 x 10^6 cells) were injected meaning that the initial degree of T cell lymphopenia would be comparable between hosts receiving cells from Lck^{Ind} $Fyn^{-/-}$ and Lck^{Ind} Fyn WT mice. Furthermore, induction of IBD in Rag-$1^{-/-}$ mice does not normally occur when transferring total LN preps as the presence of regulatory cells in the T cell memory pool provide disease protection in normal circumstances (Sakaguchi *et al.*, 1995). To address the impact of Dox dose on the manifestation of the disease the recipients were split into a further two groups and fed either 1 mg/g or 3 mg/g Dox food. A further advantage of this system is that the transferred T cells will activate and proliferate in response to the lymphopenic environment of the Rag-$1^{-/-}$ mouse. Therefore we would be comparing cells that have been activated in exactly the same manner and environment the only difference being the presence or the absence of Fyn.

Regardless of the concentration of Dox food in the diet, Rag-$1^{-/-}$ mice that received Lck^{Ind} $Fyn^{-/-}$ cells lost weight over an 8 week period (Figure 6.10A). In contrast Lck^{Ind} Fyn sufficient cells failed to induce any wasting disease in the recipient animals with either 1 mg/g or 3 mg/g Dox administration. After 24 hours post cell transfer, mice were bled and the frequency of CD4+ PBL was determined (Figure 6.10B) in order to determine the success of the transfer and to

provide an idea of initial T cell numbers. It is evident from Figure 6.10B that at this time T cell frequencies were comparable in the blood of the recipient animals. However, with time the frequency of CD4+ PBL increased in all mice, with a greater increase observed in animals that had received $Lck^{Ind}Fyn^{-/-}$ cells.

When these mice were culled analysis of the periphery revealed that cells from Lck^{Ind} $Fyn^{-/-}$ mice had expanded more than Lck^{Ind} Fyn sufficient cells, again regardless of Dox dose (Figure 6.10C). This increase in T cell numbers was particularly evident in the spleens of these mice. Interestingly, the Lck^{Ind} $Fyn^{-/-}$ mice fed 3 mg/g Dox food had elevated T cell numbers in the MLN, but not in the PLN, whereas animals on a 1 mg/g Dox diet showed increased numbers at both sites. Collectively these data show that whatever was driving the weight loss and T cell proliferation was cell intrinsic as it could be transferred to Rag-$1^{-/-}$ recipients. Also differences in initial T cell numbers or Dox diet were unlikely to be the driving factor behind the disease in $Lck^{Ind}Fyn^{-/-}$ mice.

As shown in Figure 6.6, a higher frequency of CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice were able to make IL-2 and, especially, IFNγ after *ex vivo* restimulation compared to B6 mice. Also there were differences in the pattern of CD62L and CD45RB expression and CD5 levels. As mentioned however, we were comparing two very different populations of cells, one activated and the other naïve. In our transfer system we were activating both groups of cells in the same manner and thus making the cell populations more equivalent. To this end we assessed *ex vivo* cytokine production (Figure 6.10D upper panels), CD62L / CD45RB (middle panels), and CD5 (lower panels) expression. Figure 6.10D shows that cells

expressing reduced Lck and WT levels of Fyn, when activated in response to lymphopenia, were mainly able to produce only IL-2 after restimulation with very few cells producing IFNγ. In contrast to this, cells lacking Fyn produced both IFNγ and IL-2 but there was a particularly striking bias toward cells only producing IFNγ.

When CD62L and CD45RB expression was assessed it was evident that Lck^{Ind} cells expressing Fyn had down modulated levels of both molecules in response to lymphopenic activation. In contrast Lck^{Ind} $Fyn^{-/-}$ cells down-modulated CD62L levels but the majority maintained CD45RB expression. Furthermore, analysis of CD5 expression revealed that Lck^{Ind} $Fyn^{-/-}$ cells had a 2.5-fold reduction in the CD5 MFI. In conclusion, these data showed that the phenotype of the Lck^{Ind} $Fyn^{-/-}$ cells was not simply due to comparing activated with naïve cells. It is also suggested that the activation induced differentiation programme of Lck^{Ind} $Fyn^{-/-}$ cells was different from Lck^{Ind} fyn sufficient cells. Interestingly, the phenotype of the CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice resembled effector-memory cells in that they are $CD45RB^{hi}$, $CD62L^{lo}$ (Hasegawa *et al.*, 2004) and mainly IFNγ producers (Blander *et al.*, 2003; Wang and Mosmann, 2001).

6.11 Transfer of $CD44^{hi}$ and $CD44^{lo}$ CD4 Lck^{Ind} $Fyn^{-/-}$ cells is capable of inducing disease

Having shown that the wasting disease was in fact cell intrinsic we wanted to determine exactly which cells were capable of conferring sickness. In the previous experiments total LN containing CD4 and CD8 T cells, as well as B cells

was used as the source of material for the transfer into recipient mice. As sick mice showed expansion of all of these cell populations to various degrees we could not determine which cell types were required for disease induction. From previous data we reasoned that CD4 cell expansion and the phenotypic changes observed in this population from sick Lck^{Ind} $Fyn^{-/-}$ mice could be a major factor in the disease. To test this theory we purified CD4 T cells from the PLN of healthy 4 week old Lck^{Ind} $Fyn^{-/-}$ mice and Lck^{Ind} Fyn sufficient mice to ~ 96% purity in both groups (data not shown). It should be mentioned that all mice were weaned on a 3 mg/g Dox diet. Equal numbers of purified CD4 cells (1 x 10^6) were injected i.v into Rag-$1^{-/-}$ mice. Moreover, as well as total CD4, another two groups of mice received 1 x 10^6 of either naïve ($CD44^{lo}$) or memory ($CD44^{hi}$) purified CD4 cells from healthy Lck^{Ind} $Fyn^{-/-}$ donor mice. Purity in the memory group was ~99.9%, however there was ~10% CD44hi cells remaining in the naive group (data not shown). Again it should be stressed that transfer of comparatively large numbers of naïve WT cells does not normally induce IBD in Rag-$1^{-/-}$ mice on a B6 background (data not shown). IBD is usually induced by transferring 1-5 x 10^5 naïve T cells (Sakaguchi *et al.*, 1995). In all cases mice were fed 3 mg/g Dox food to maintain transgene driven Lck expression. At 24 hours post transfer, recipient mice were bled and the frequency of $CD4^+$ PBL was assessed. Figure 6.11A shows the CD44 profile of $CD4^+$ PBL from all four groups of mice. From these data it was evident that there was an increased percentage of $CD44^{hi}$ cells in the recipients that received total CD4 cells from Lck^{Ind} $Fyn^{-/-}$ donors compared to Lck^{Ind} Fyn sufficient mice. As expected there was no $CD44^{lo}$ cells evident in the blood of mice that received memory CD4 cells, however there was a percentage of $CD44^{hi}$ cells evident in the blood of recipients injected with naïve Lck^{Ind} $Fyn^{-/-}$

cells. In all groups the initial frequency of total CD4 T cells was comparable (data not shown).

When the weights of the recipient mice were monitored it became evident that the wasting disease manifested 8-12 weeks post transfer (Figure 6.11B) only in animals that received either total CD4 cells or memory CD4 cells from Lck^{Ind} $Fyn^{-/-}$ donor mice. Within this time frame, mice that received naïve CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice remained healthy and actually increased in weight, as did mice that received total CD4 from Lck^{Ind} Fyn WT donors. However, after 15 weeks post transfer, animals that received the naïve CD4 cells from Lck^{Ind} $Fyn^{-/-}$ animals began to rapidly lose weight and were culled.

We next sought to determine the phenotype of the CD4 cells from the four groups of mice. In all mice 100% of CD4 cells became $CD44^{hi}$ due to lymphopenic activation (data not shown). Figure 6.11C (upper panel) shows that the majority of CD4 cells from Lck^{Ind} Fyn sufficient controls converted to a $CD62L^{lo}$ $CD45RB^{lo}$ phenotype after transfer. In contrast CD4 Lck^{Ind} $Fyn^{-/-}$ cells again lost CD62L expression but the majority remained $CD45RB^{hi}$. This phenotype was observed regardless of the starting population of Lck^{Ind} $Fyn^{-/-}$ cells transferred. CD5 expression was also assessed and found to be reduced on the surface of CD4 cells in all groups of Lck^{Ind} $Fyn^{-/-}$ cells transferred compared to Lck^{Ind} Fyn sufficient cells. As noted in figure 6.6, the decrease in MFI seemed to be due to the appearance of a distinctive $CD5^{lo}$ cell population.

$CD44^{hi}$ CD4 cells can be subdivided into two separate groups depending on the expression of CD62L and CD45RB. $CD44^{hi}$ $CD62L^{lo}$, $CD45RB^{lo}$ CD4 cells are considered to be central memory cells and $CD44^{hi}$ $CD62L^{lo}$ $CD45RB^{hi}$ cells are thought to be effector-memory cells (Hasegawa *et al.*, 2004; Dutton *et al.*, 1998). In the sick mice we seemed to have an accumulation of effector-memory type CD4 cells as a large proportion expressed CD45RB (Figure 6.6D. Furthermore, when we transferred the Lck^{Ind} $Fyn^{-/-}$ cells *in vivo* they seem to preferentially become effector-memory type cells (Figure 6.10D). Another factor that distinguishes central memory from effector memory is that effector memory cells can produce IFNγ upon recall stimulation. From the data in Figure 6.6B/C it was clear that $CD44^{hi}$ cells were able to make IFNγ upon recall stimulation, also when the cells from Lck^{Ind} $Fyn^{-/-}$ mice were activated *in vivo* they preferentially secreted only IFNγ and very little IL-2 (Figure 6.10D). Therefore we wanted to determine if the major cell population able to make IFNγ from Lck^{Ind} $Fyn^{-/-}$ mice were also $CD62L^{lo}$, $CD45RB^{hi}$, and thus effector-memory-like. Figure 6.11D shows the IL-2/ IFNγ staining profile of $CD45RB^{lo}$ (left panels) and $CD45RB^{hi}$ (right panels) CD4 $CD44^{hi}$ cells from Lck^{Ind} $Fyn^{-/-}$ (lower panels) and Lck^{Ind} mice expressing Fyn (upper panel) after transfer into Rag-$1^{-/-}$ recipients. It is evident that Lck^{Ind} $Fyn^{-/-}$ $CD45RB^{hi}$ CD4 cells were mainly producing IFNγ, with very few cells only able to make IL-2 within this population. In contrast, a percentage of the $CD45RB^{lo}$ population was able to make just IL-2, although there were cells able to make IFNγ. In the Fyn sufficient group, the $CD45RB^{hi}$ cells also had a higher percentage of IFNγ single-producer cells compared to the $CD45RB^{lo}$ cells, however the majority were able to produce IL-2 only. Collectively these data support the idea that activation of cells from Lck^{Ind} $Fyn^{-/-}$ mice leads to the

accumulation of effector-memory like CD62L^{lo}, CD45RB^{hi} cells that are preferentially able to only make IFNγ.

6.12 Discussion

The aim of this chapter was to characterise the wasting disease observed in mice expressing reduced Lck and no Fyn. By monitoring the weights of cohorts of mice representing all possible combinations of Lck and Fyn, we confirmed that sickness was only evident in Dox fed Lck^{Ind} Fyn$^{-/-}$ mice. When 20% of initial body mass was lost, mice were culled for analysis, this occurred anywhere between 4-12 weeks of age. Analysis of the GI tract revealed swelling of the upper regions and cellular infiltrate within the lumen of the villi. The PLN, MLN and spleens of these mice were enlarged due to an accumulation of effector/memory type CD4 cells that had lowered CD5 expression and could make IFNγ upon recall stimulation. There was also a degree of B cell disregulation that led to increased B cell numbers in the MLN and PLN. Moreover, there were indications of autoimmunity as we could detect both ANA and IgE in serum of sick mice. We were able to regulate disease initiation and progression by administering or removing Dox from the diet of the mice, showing that the expression of Lck was essential to the disease state. The disorder was not driven by the Lck^{Ind} Fyn$^{-/-}$ mice being more lymphopenic than Fyn sufficient Lck^{Ind} mice, nor was it due to differences in the dosage of Dox administered to the groups of mice. This was shown in transfer experiments using equal numbers of T cells from Lck^{Ind} Fyn$^{-/-}$ or Lck^{Ind} Fyn WT mice into Rag-1$^{-/-}$ hosts. We were able to induce wasting disease only in Rag-1$^{-/-}$ hosts that received Lck^{Ind} Fyn$^{-/-}$ cells, and this occurred with either Dox dosage. Furthermore, we showed that the

wasting disease could be conferred specifically by transferring either CD4 naïve or memory-like cells from Lck^{Ind} $Fyn^{-/-}$ mice. Activation of Lck^{Ind} $Fyn^{-/-}$ CD4 cells in response to lymphopenia led to the accumulation of effector/memory type cells, whereas Lck^{Ind} cells seemed to become more resting memory-like. This observation was based on the fact that Lck^{Ind} $Fyn^{-/-}$ cells became $CD44^{hi}$ $CD62L^{lo}$ $CD45RB^{hi}$ and were able to produce IFNγ. In contrast Lck^{Ind} cells also became $CD44^{hi}$ $CD62L^{lo}$, but were $CD45RB^{lo}$ and produced mainly IL-2.

The reason for the drastic weight loss observed in the Lck^{Ind} $Fyn^{-/-}$ mice seemed to result from inflammation of the regions of the GI tract. In more classical models of IBD, the inflammation is normally restricted to the colon (Strober *et al.*, 2002), and not the upper regions. When we have induced IBD in Rag-$1^{-/-}$ mice by transferring small numbers of naïve CD4 cells from WT mice, as expected animals lost weight due to diarrhoea and colonic inflammation (data not shown). However, when we fed these recipient mice Dox food, we still observed IBD, but the site of swelling resembled that seen in Lck^{Ind} $Fyn^{-/-}$ mice. We feel that although phenotypically distinct, the disease observed in the Lck^{Ind} $Fyn^{-/-}$ mice may still be related to classical IBD as the site of inflammation may differ simply due to the administration of Dox. IBD-like disorders in general are a direct result of mucosal inflammation in response to commensal gut antigens (Strober *et al.*, 2002). As Dox is antibiotic, it could change the nature of the disease and the site of inflammation by altering the balance of bacteria in the GI tract.

IBD can be subdivided into two categories depending on the level of immune disregulation responsible for causing it. Type I disorders arise from autoagression

of gut T cells that lead to a loss of tolerance to commensal gut antigens (Strober *et al.*, 2002). An example of a Type I disorder is IBD induced by the transfer of cells from mice expressing a STAT4 transgene into SCID recipients. The over expression of STAT4 renders the T cells hyper-responsive to IL-12 signalling, breaking tolerance to gut antigens (Wirtz *et al.*, 1999). Furthermore, Type I disorders are in general associated with Th1 responses (Strober *et al.*, 2002). This is most likely because the bacterial agents that include superantigens, such as SEA, and mitogens like LPS or CpGs will induce IL-12 production, leading to a preference for Th1 responses (Strober *et al.*, 2002). In contrast, a Type II disorder is thought to arise from a failure in the regulatory mechanisms that would usually control an otherwise normal effector response (Strober *et al.*, 2002). Transferring naïve CD4 cells into SCID mice is an example of a type II disorder as IBD is induced because there are no regulatory cells normally present in the memory population (Powrie *et al.*, 1994), allowing the cells to proliferate unchecked.

There is evidence to suggest our model of IBD has elements of a Type I mucosal inflammation disorder. Firstly, it was interesting that such a comparatively high number of naive CD4 cells from the Lck^{Ind} $Fyn^{-/-}$ mice could induce the IBD-like syndrome upon transfer. In typical SCID IBD models involving B6 mice that are driven by type II immunological defects (an absence of appropriate regulatory mechanimsms), there is a direct correlation between the degree of lymphopenic expansion and the severity of IBD induction. Transferring small numbers (1-2 $x10^5$) of naïve CD4 cells normally induces rapid disease as these relatively few T cells undergo significant proliferation in order to fill the space in the lymphopenic host. This proliferation and activation is normally controlled by T_{reg} cells within

the memory population (Sakaguchi *et al.*, 1995; Powrie *et al.*, 1994). In contrast, larger numbers of transferred naïve CD4 cells do not undergo the same degree of expansion and fail to induce IBD in empty hosts. Therefore we may have expected that disease progression should not have occurred after the transfer of such a comparatively high number of naïve CD4 T cells. The fact that we did observe disease suggested that there was something intrinsically autoagressive about the naïve T cells derived from the Lck^{Ind} $Fyn^{-/-}$ mice that was not simply due to an absence of T_{reg} populations. It is also evident from our data that the CD4 $CD44^{hi}$ cells that also contain the putative regulatory cell populations seemed to induce more rapid disease in the mice instead of conferring protection. This result suggested that the rate-limiting step for disease induction could have been the activation and conversion of naïve CD4 cells to $CD44^{hi}$ cells, but also that the T_{reg} cells were unable to suppress.

Evidence that the CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice were somehow disregulated came from the observation that when we restimulated them *ex vivo*, we were able to detect the potential to produce IFNγ. This was not observed in cells from Lck^{Ind} mice. The major IFNγ producing cell population was $CD44^{hi}$ $CD62L^{lo}$ $CD45RB^{hi}$, suggesting that these are effector/memory type CD4 cells (Hasegawa *et al.*, 2004; Dutton *et al.*, 1998). Moreover, we could transfer the disease to Rag-$1^{-/-}$ recipients by virtue of these cells alone. IFNγ has been shown to increases the severity of colitis in SCID models. There is also evidence that the deletion of CD4 cells *in vivo* can ameliorate mucosal inflammation (Okamoto *et al.*, 1999). Moreover, in SCID models of IBD, it is generally $CD45RB^{hi}$ CD4 cells that are transferred in order to induce disease (Claesson *et al.*, 1999). These facts highlight the

important contribution that disregulated CD4 function could have to mucosal inflammation in the Lck^{Ind} $Fyn^{-/-}$ mice. Furthermore work by Stein et al suggested that there may have been defects in the deletion of autoreactive T cells in $Fyn^{-/-}$ mice (Stein *et al.*, 1992). This could have been exacerbated by reducing Lck levels leading to the escape of autoreactive T cells from deletion in the thymus. Collectively these data suggested that disease manifestation was intrinsic to the CD4 cell population and therefore had elements resembling a Type I model of mucosal inflammation.

As discussed in Chapter 5, in the absence of Fyn, CD8 cells may mount a more prolonged primary response by virtue of their inability to efficiently downmodulate TCR signals. In the Lck^{Ind} $Fyn^{-/-}$ mice, we may be observing a more severe manifestation of the same phenomenon where disregulated T cell activation in response to self-antigens led to an accumulation of CD4 effector/memory phase of differentiation. Certainly, when we transferred Fyn WT and $Fyn^{-/-}$ Lck^{Ind} cells into empty hosts, both groups became activated and underwent lymphopenic expansion. However, while Lck^{Ind} Fyn WT cells became central memory-like cells, the Lck^{Ind} $Fyn^{-/-}$ cells seemed to remain in a state of continued activation.

From our data it was clear that low levels of Lck expression in the absence of Fyn was an absolute requirement for disease induction. However, when we removed sick mice from Dox it was possible to ameliorate the disease and even halt the weight loss. Therefore, we could conclude from these data that we needed continual Lck expression to maintain the disease state. This suggested to us that

the reduced Lck levels were able to transmit activation signals, but were unable to regulate the response without Fyn. To try and explain these data we propose the model shown in Figure 6.12. At WT levels of expression, Fyn and Lck seem to contribute to the proliferative and regulatory signals, but Lck may be better suited to driving proliferative signals, and Fyn may be better at downregulating the response (see Figure 6.12A and B). This conclusion is based on the phenotype of the respective knockout mice, where the loss of Lck leads to inefficient activation whereas the loss of Fyn leads to inefficient regulation of the response. In the Lck^{Ind} system, the reduced Lck expression does not seem to compromise the ability of the cells to become activated, nor to terminate the response if Fyn is also present. However, the low level of Lck seems to be unable to compensate for the loss of Fyn in terms of downregulating a response (see Figure 6.12C). This is an interesting model, as it suggests that Lck and Fyn may not have unique functions in the true sense of the word, but instead possess preferential roles in controlling T cell responses.

There is one conflicting observation to this model as we have observed upregulation of Lck levels in activated Lck^{Ind} $Fyn^{-/-}$ cells following ~72 of *in vitro* activation (Lovatt et al manuscript submitted). Therefore it is also a possibility that the activation-induced upregulation of Lck in these cells is responsible for breaking tolerance as the activation threshold of Lck^{Ind} $Fyn^{-/-}$ cells may have been set with respect to the low Lck levels present in a resting state. However it should also be noted that although significantly upregulated, the level of Lck is still lower than in a WT cell (~75%). When the Lck^{Ind} $Fyn^{-/-}$ cells are developing in the thymus, they express Lck levels comparable to WT (see Figure 6.1B). However

we have not established whether Lck trangene levels are downregulated upon export to the periphery, or if this occurs in the transition from DP to SP. SP cells are still thought to undergo proliferation and alterations in surface marker expression in medullary areas of the thymus for ~2 weeks (Gabor *et al.*, 1997; Ernst *et al.*, 1995). Therefore it is possible that the activation threshold of a T cell is set in some way during this period of SP thymocyte development. Interestingly, Fyn expression has been shown to significantly upregulate between the DP and SP stages (Olszowy *et al.*, 1995), and if Fyn is important in regulating a T cell response, this may be important to the activation threshold of the T cell.

As mentioned previusoly Fyn may regulate the formation of the PAG/Csk/PEP inhibitory complex but there is also evidence that Lck may be able to phosphorylate PAG also (Brdicka *et al.*, 2000). Therefore it is possible that the reason for the disrgeulation of the Lck^{Ind} $Fyn^{-/-}$ T cells is due to a failure to assemble the PAG/Csk/PEP complex as there is no Fyn and reduced Lck levels. Interestingly an increase of effector/memory CD4 cells has also been reported in mice that lack the phosphatase PEP (Hasegawa *et al.*, 2004). This suggests there could be some common mechanism between the defect in PEP deficient mice and our Lck^{Ind} $Fyn^{-/-}$ mice. However, there are differences between the phenotype of the Lck^{Ind} $Fyn^{-/-}$ mice and $PEP^{-/-}$ mice as there was no evidence of IBD or ANA reported by Hasegawa and colleagues. One reason for this lack of autoimmune phenotype is that in $PEP^{-/-}$ mice, PAG Tyr 317 phosphorylation would presumably be unaffected meaning that Csk could still associate with PAG and phosphorylate the C-termini residues on Lck and Fyn. Moreover, in the $PEP^{-/-}$ mice, the presence of other phosphatases such as PTP-PEST may be able to compensate for the loss

of PEP (Davidson and Veillette, 2001) rendering the immune disregulation less severe. In our Lck^{Ind} $Fyn^{-/-}$ mice, this whole complex may be completely absent due to no Tyr 317 phosphorylation leading to the inappropriate and disregulated activation of T cells in these animals. We are planning to look at the status of PAG Tyr 317 in the cells from Lck^{Ind} $Fyn^{-/-}$ mice to address this question. However it should also be noted that $PAG^{-/-}$ mice remain healthy (Dr Lindquist, personal communication), therefore the disregulation of PAG is presumably not the only molecule possibly affected in Lck^{Ind} $Fyn^{-/-}$ mice.

As the phenotype of both $PAG^{-/-}$ and $PEP^{-/-}$ mice differ from what we see it is also possible that other immunomodulatory mechanisms may be defective in the Lck^{Ind} $Fyn^{-/-}$ mice. As discussed in Chapter 3, Lck and Fyn have been implicated in the signalling of CTLA-4 (Hu *et al.*, 2001). Furthermore, $CTLA\text{-}4^{-/-}$ mice also suffer from a lymphoproliferative disorder (Khattri *et al.*, 1999; Shrikant *et al.*, 1999). However there are differences wherby the CD4 cells generated by this response are Th2-like, and secrete IL-4 and IL-5 upon *ex vivo* restimulation (Khattri *et al.*, 1999). In contrast, a major proportion of CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice were more Th1-like by virtue of their ability to produce IFNγ. However we could not exclude that some Th2 cells were present as we were able to detect IgE in the serum of the Lck^{Ind} $Fyn^{-/-}$ mice. Furthermore, as discussed in Chapter 5, Fyn has been shown to mediate signalling downstream of the immunoinhibitory receptor SLAM (Chan *et al.*, 2003) through the linker molecules SAP. It is possible that the disease observed in the Lck^{Ind} $Fyn^{-/-}$ mice is influenced, in part, by defects in SLAM mediated signalling as $SAP^{-/-}$ cells show increased ability to produce IFNγ (Czar *et al.*, 2001). However, recent work has shown that these cells are not

predisposed to become Th1 cells, rather that the production of Th2 cytokines is defective in these mice (Cannons *et al.*, 2004; Davidson *et al.*, 2004). Furthermore $SAP^{-/-}$ mice show no evidence of autoimmune disease (Czar *et al.*, 2001), suggesting that it is unlikely that disregulated SLAM signalling is the sole cause of the autoimmune disorder in Lck^{Ind} $Fyn^{-/-}$ mice. We cannot rule out that the reduction in Lck levels combined with ablated Fyn expression is altering the function of T cell signalling receptors other than the TCR, and that disregulation of multiple pathways is responsible for the disease. We plan to address these question in future experiments. For example, if disregulated TCR signals were solely responsible for disease induction, then we may not expect CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice to induce disease in MHC-class II deficient hosts.

As well as an effect on T cell function, we also noted that B cell numbers were often increased in Lck^{Ind} $Fyn^{-/-}$ mice. Furthermore, we also noted that we could detect both IgE and ANA in the serum of Lck^{Ind} $Fyn^{-/-}$ mice, correlating with disease incidence. From the transfer experiments, it was evident that B cells were not required for disease induction, as the purified CD4 T cells conferred weight loss alone. Interestingly however, when B and T cells were co-transferred into recipient mice, disease induction occurred on average at 9 weeks, but when T cells were injected alone, disease manifestation was evident between 10-12 weeks. Although ANA have been reported in IBD models, it is thought that B cells are generally not important to the disorder (Strober *et al.*, 2002). However, it is possible that the presence of B cells was able to accelerate the disease, however this would need to be confirmed by further experiments as the difference in induction time is subtle.

As the CD4 cells seemed to be the driving factor behind the disease, it seemed likely that the B cells changes were a secondary effect. Certainly, CD4 cells can influence B cell function through the action of secreted cytokines including IL-4 and IL-13 that can induce immunoglobulin (Ig) class switching (Vercelli, 1995). Furthermore, CD40L on CD4 T cells can interact with CD40 on the surface of the B cell and induce proliferation, Ig production and Ig class switching (Bishop and Hostager, 2003). The interaction of activated CD4 T cells with B cells are thought to occur in specialised structures within the B cell follicles of secondary lymphoid tissue. These areas are termed germinal centres (GC). Moreover, the spontaneous formation of GC has been reported in the spleens of mice that suffer from autoimmune disorders such as type I diabetes and a lupus like syndrome (Luzina *et al.*, 2001). Considering these data it may be of interest to look for the spontaneous formation of GC in the spleens Lck^{Ind} $Fyn^{-/-}$ mice. B cells within these areas are generally $B220^{+}$ PNA^{+} $GL\text{-}7^{+}$ (Luzina *et al.*, 2001) and can be visualised using immunohistochemistry (Hasegawa *et al.*, 2004). Furthermore, it may be informative to transfer CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice into $TCR\alpha^{-/-}$ mice and look at the influence these transferred T cells have on B cell function. Conversley, we could also transfer B cells from Lck^{Ind} $Fyn^{-/-}$ mice into μMT mice that lack B cells and see if we can induce disease. These experiments would answer if B cell disregulation in the Lck^{Ind} $Fyn^{-/-}$ mice was a direct result of altered T cell function. Lastly, it may be informative to look at the expression of CD40L on the CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice. If CD40L levels are increased in Lck^{Ind} $Fyn^{-/-}$ mice it may explain why the B cell population is disregulated.

Although data showing phenotypic differences in the CD4 population may suggest that the IBD-like disorder we observe in Lck^{Ind} $Fyn^{-/-}$ mice is a Type I model of mucosal inflammation, it is also possible that it could be a class II disorder, or even a composite of both. We could see the presence of putative CD4 $CD25^{+}$ T_{reg} cells in our healthy Lck^{Ind} $Fyn^{-/-}$ mice at similar frequencies to B6 controls. However, what is not clear is whether the IBD was caused by a lack of suppressive function, or because the CD4 cell expansion overwhelms their ability to regulate. In terms of the former idea, studies have shown that these cells may exert effects by cell-cell contacts (Nakamura *et al.*, 2001; Thornton and Shevach, 1998) that would presumably involve some form of receptor engagement that could require Fyn and Lck functions. Therefore, the loss of Fyn, and the reduction in Lck levels may prevent the CD4 $CD25^{+}$ T_{reg} cells from suppressing. Although our data shows that the number of CD4 $CD25^{+}$ cells expanded with disease, it was not clear if these cells represented newly activated CD4 cells, or whether they were infact T_{reg} cells. In any case, it is clear that these cells fail to control the immune response in the Lck^{Ind} $Fyn^{-/-}$ mice. We are currently addressing the phenotype and function of T_{reg} cells from Lck^{Ind} $Fyn^{-/-}$ mice to determine of they possess suppressive capabilities. Furthermore, it may be interesting to transfer T_{reg} cells from WT mice into Lck^{Ind} $Fyn^{-/-}$ mice showing signs of sickness to determine if disease could be ameliorated.

It is possible that the accumulation of these activated effector/memory type $CD44^{hi}$ $CD62L^{lo}$ $CD45RB^{hi}$ CD4 cells in sick mice is influenced by defective AICD mechanisms that would normally facilitate the removal these cells (Green *et al.*, 2003). For example, studies suggest that Lck is essential to the induction of

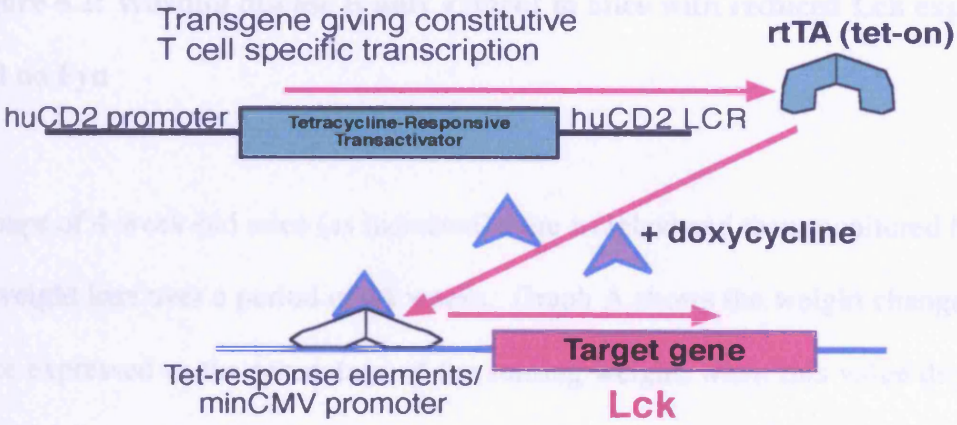
AICD in cycling T cells after ligation of the TCR (Yu *et al.*, 2004). Furthermore, as mentioned in Chapter 3, Fyn has been shown to physically associate with the cytoplasmic tail of Fas (Atkinson *et al.*, 1996), and there is a report suggesting $Fyn^{-/-}$ cells are resistant to Fas-mediated AICD (Ricci *et al.*, 2001). Certainly, *lpr/lpr* mice that have non-functional Fas, and *gld/gld* mice with a FasL mutation both suffer from autoimmunity and lymphoproliferation (Lynch *et al.*, 1994; Takahashi *et al.*, 1994; Watanabe-Fukunaga *et al.*, 1992). Therefore, it may be of interest to induce AICD in these cells *ex vivo* with antibodies against the Fas receptor, or using anti-CD3 stimulation of pre-activated T cells.

Finally, another intruiging pehonotype that requires further analysis is the observation that Lck^{Ind} $Fyn^{-/-}$ mice often presented with a constant shaking of the head, and also the manifestation of varying degrees of hind leg paralysis (data not shown). This suggested that there may also be a neurological component to the disease that resembles experimental autoimmune encephalomyelitis (EAE). As EAE is though to be caused by infiltrating CD4 cells (Segal, 2003), it will be of interest to assess the presence of CD4 cells in brain sections of symptomatic Lck^{Ind} $Fyn^{-/-}$ mice using immunohistochemistry.

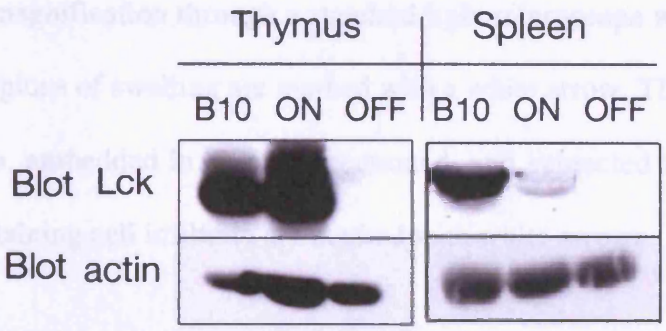
Figure 6.1: Overview of the Lck^{Ind} system and protein expression patterns

Lck expression is regulated in the lymphoid system of our mice by the tetracycline-responsive gene induction system. Briefly, when the tetracycline derivative Doxycycline is present, the constitutively expressed rtTA protein is able to bind to the tet-response element upstream of the minimal CMV promoter and drive expression of the Lck transgene (A). While expression of Lck using this system is comparable to WT Lck levels in the thymus (B, left panels), peripheral expression is reduced to ~20% of WT (B, right panels). When Lck^{Ind} $Fyn^{-/-}$ mice were fed 3 mg/g Dox food, they had to be culled due to rapid weight loss (C). However, Dox food did not affect the weight of mice of any other genetic background. Figures A and B are adapted from data supplied by Dr Ben Seddon and Dr Matthew Lovatt.

A


Transgene giving constitutive
T cell specific transcription
rtTA (tet-on)
huCD2 promoter
Tetracycline-Responsive Transactivator
huCD2 LCR
+ doxycycline
Target gene
Tet-response elements/
minCMV promoter
Lck

B


Thymus
Spleen
B10 ON OFF
B10 ON OFF
Blot Lck
Blot actin

C

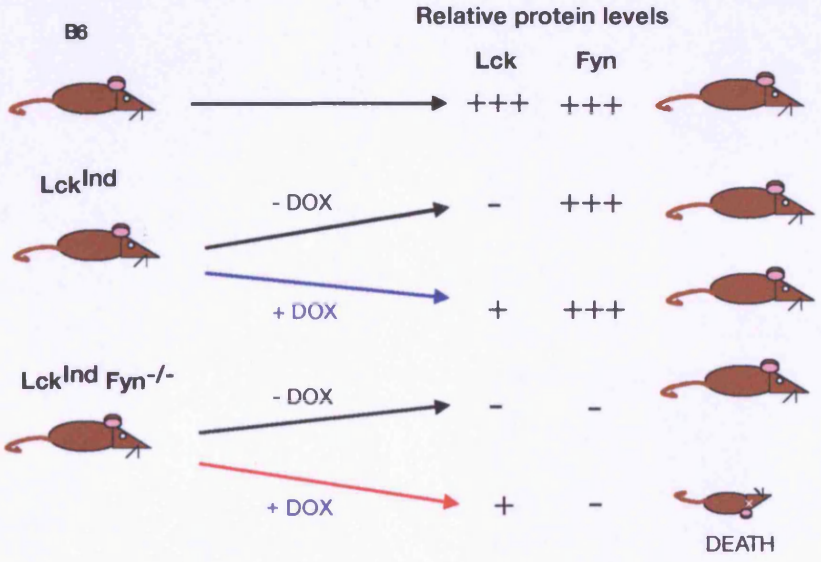

B6
Relative protein levels
Lck
Fyn
+++
+++
Lck^Ind
- DOX
-
+++
+ DOX
+
+++
Lck^Ind Fyn^-/-
- DOX
-
-
+ DOX
+
-
DEATH

Figure 6.2: Wasting disease is only evident in mice with reduced Lck expression and no Fyn

Groups of 4-week-old mice (as indicated) were weighed and then monitored for signs of weight loss over a period of 24 weeks. Graph A shows the weight changes in the mice expressed as the percentage of the starting weight, when this value dropped to ~85%, mice were culled for analysis. The GI tract was removed and photographed (B) under 10x magnification through a standard light microscope with digital camera attachment. Regions of swelling are marked with a white arrow. The gut was fixed in Bouins solution, embedded in paraffin, sectioned, and subjected to H & E staining (C). Areas containing cell infiltrate are marked with white arrows.

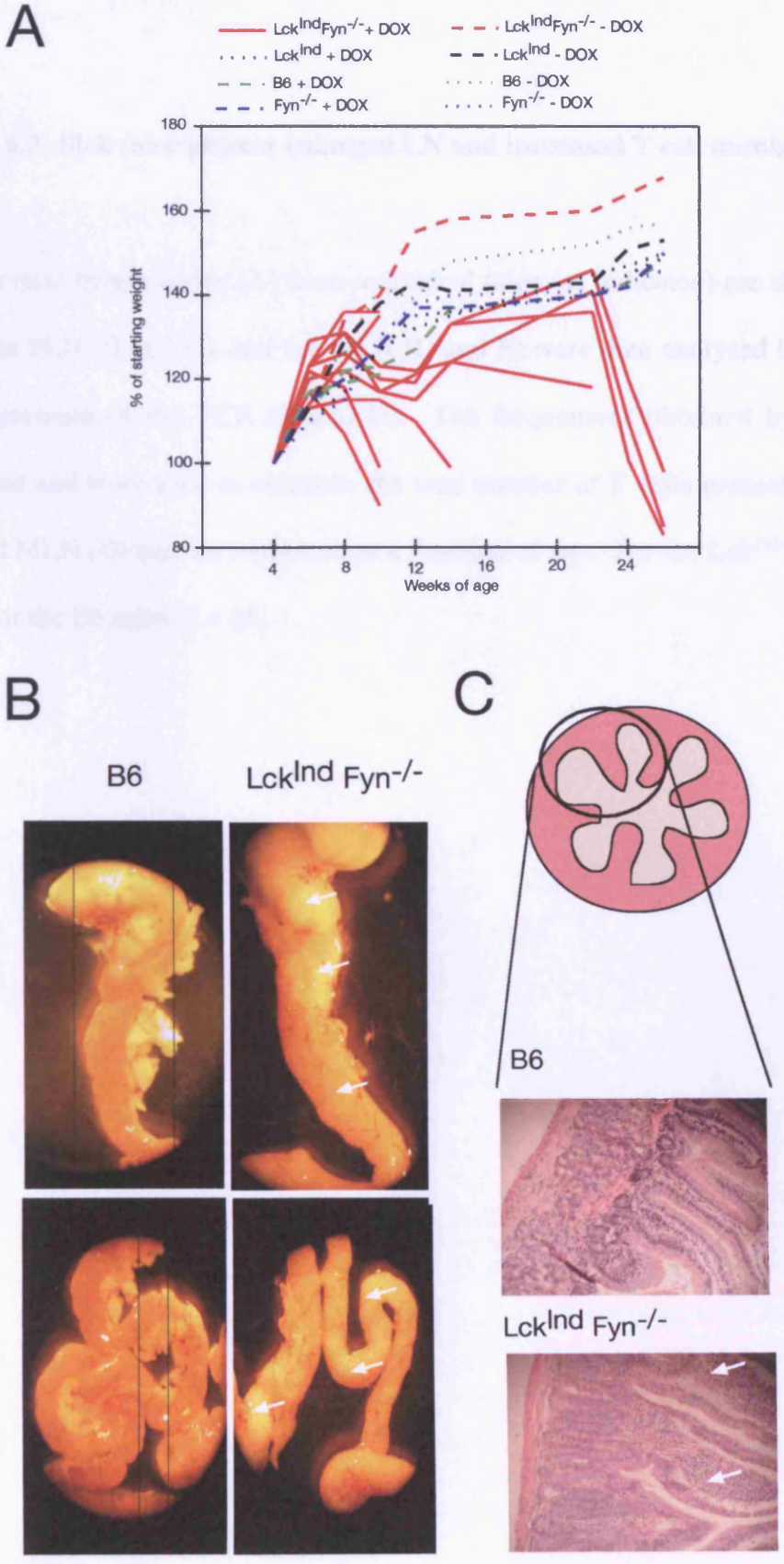
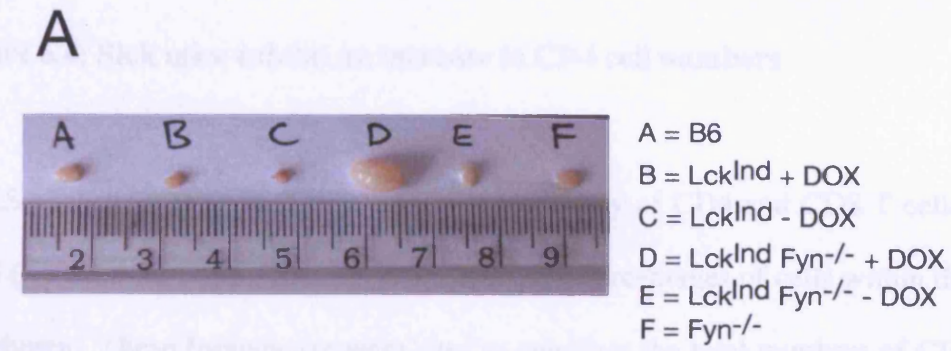
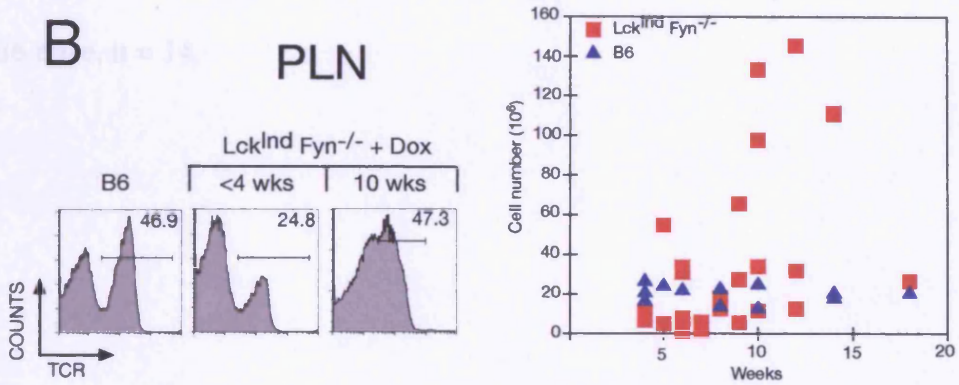

A
Lck^Ind Fyn^-/- + DOX
Lck^Ind + DOX
B6 + DOX
Fyn^-/- + DOX
Lck^Ind Fyn^-/- - DOX
Lck^Ind - DOX
B6 - DOX
Fyn^-/- - DOX
% of starting weight
180
160
140
120
100
80
4
8
12
16
20
24
Weeks of age
B
B6
Lck^Ind Fyn^-/-
C
B6
Lck^Ind Fyn^-/-

Figure 6.3: Sick mice possess enlarged LN and increased T cell numbers

The cervical lymph nodes (A) from individual mice (as indicated) are shown. Cells from the PLN (B and C), and the MLN (D and E) were then analysed by FACS for the expression of the TCR (B and D). The frequencies obtained by FACS are indicated and were used to calculate the total number of T cells present in the PLN (C) and MLN (D) and are expressed as a function of age. For the $Lck^{Ind}Fyn^{-/-}$ mice n = 24; for the B6 mice, n = 14.


A
A B C D E F
2 3 4 5 6 7 8 9
A = B6
B = Lck^Ind + DOX
C = Lck^Ind - DOX
D = Lck^Ind Fyn^-/- + DOX
E = Lck^Ind Fyn^-/- - DOX
F = Fyn^-/-


B
PLN
Lck^Ind Fyn^-/- + Dox
B6
<4 wks
10 wks
46.9
24.8
47.3
COUNTS
TCR
C
Lck^Ind Fyn^-/-
B6
Cell number (10^6)
Weeks

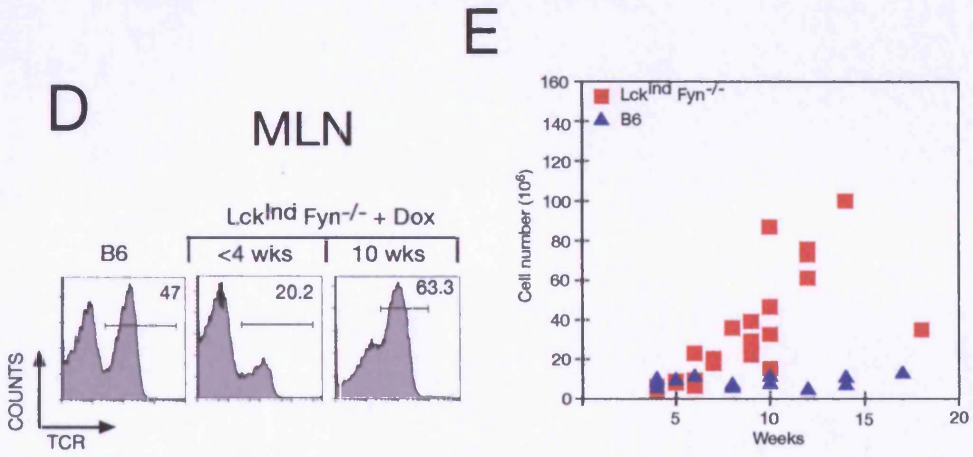

D
MLN
Lck^Ind Fyn^-/- + Dox
B6
<4 wks
10 wks
47
20.2
63.3
COUNTS
TCR
E
Lck^Ind Fyn^-/-
B6
Cell number (10^6)
Weeks

Figure 6.4: Sick mice exhibit an increase in CD4 cell numbers

FACS analysis was used to determine the frequency of CD4 and CD8 T cells in the PLN (A) and MLN (D) of the indicated mice, the percentages of cells within the gates are shown. These frequencies were used to calculate the total numbers of CD4 cells in the PLN (B) and MLN (E), as well as the number of CD8 cells in the PLN (C) and MLN (F), are shown as a function of mouse age. For the Lck^{Ind} $Fyn^{-/-}$ mice, n = 24; for the B6 mice, n = 14.

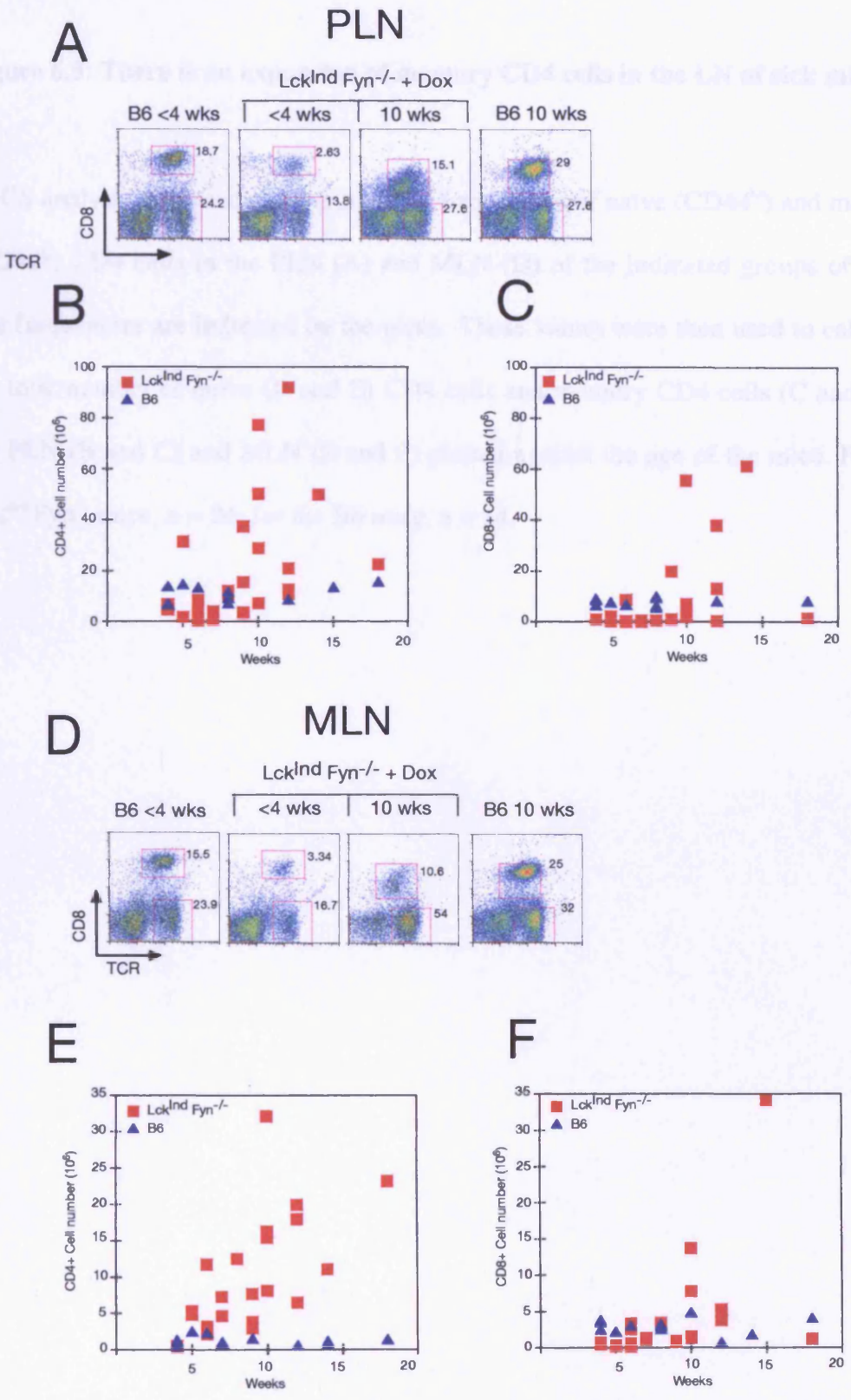

A
PLN
Lck^Ind Fyn^-/- + Dox
B6 <4 wks
<4 wks
10 wks
B6 10 wks
18.7
24.2
2.63
13.8
15.1
27.8
29
27.8
CD8
TCR
B
C
Lck^Ind Fyn^-/-
B6
CD4+ Cell number (10^6)
CD8+ Cell number (10^6)
Weeks
D
MLN
Lck^Ind Fyn^-/- + Dox
B6 <4 wks
<4 wks
10 wks
B6 10 wks
15.5
23.9
3.34
16.7
10.6
54
25
32
CD8
TCR
E
F
Lck^Ind Fyn^-/-
B6
CD4+ Cell number (10^6)
CD8+ Cell number (10^6)
Weeks

Figure 6.5: There is an expansion of memory CD4 cells in the LN of sick mice

FACS analysis was used to determine the frequencies of naïve ($CD44^{lo}$) and memory ($CD44^{hi}$) CD4 cells in the PLN (A) and MLN (D) of the indicated groups of mice. The frequencies are indicated on the plots. These values were then used to calculate the total number of naïve (B and E) CD4 cells and memory CD4 cells (C and F) in the PLN (B and C) and MLN (E and F) plotted against the age of the mice. For the $Lck^{Ind}Fyn^{-/-}$ mice, n = 24; for the B6 mice, n = 14.

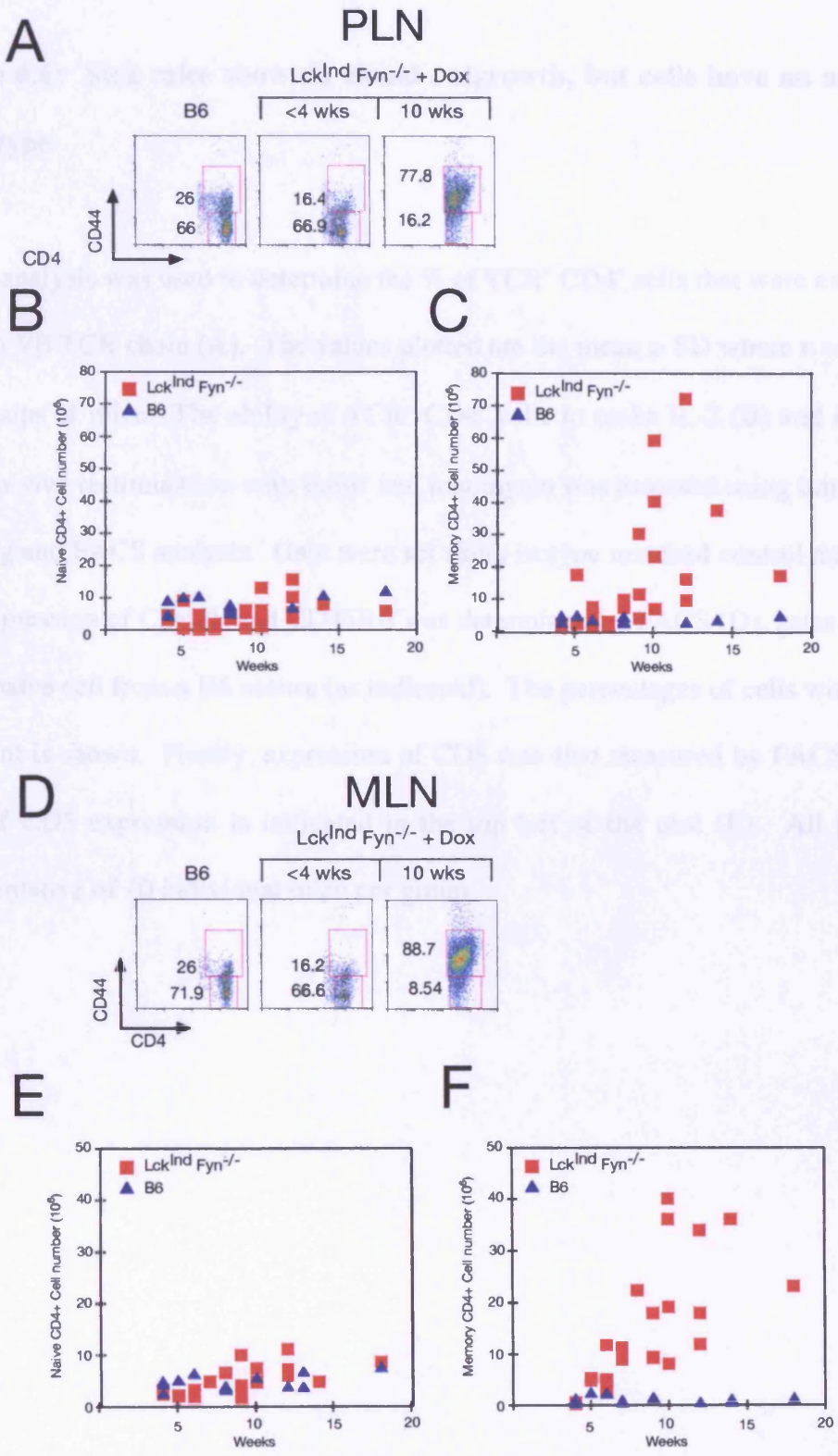

A
PLN
Lck^Ind Fyn^-/- + Dox
B6
<4 wks
10 wks
26
66
16.4
66.9
77.8
16.2
CD44
CD4
B
C
Lck^Ind Fyn^-/-
B6
Naive CD4+ Cell number (10^6)
Memory CD4+ Cell number (10^6)
Weeks
D
MLN
Lck^Ind Fyn^-/- + Dox
B6
<4 wks
10 wks
26
71.9
16.2
66.6
88.7
8.54
CD44
CD4
E
F
Lck^Ind Fyn^-/-
B6
Naive CD4+ Cell number (10^6)
Memory CD4+ Cell number (10^6)
Weeks

Figure 6.6: Sick mice show no clonal outgrowth, but cells have an activated phenotype

FACS analysis was used to determine the % of TCR^+ $CD4^+$ cells that were expressing a given Vβ TCR chain (A). The values plotted are the mean ± SD where n = 6 for all the groups of mice. The ability of TCR^+ $CD4^+$ cells to make IL-2 (B) and IFNγ (C) upon *ex vivo* restimulation with PdbU and ionomycin was assessed using intracellular staining and FACS analysis. Gate were set using isotype matched control antibodies. The expression of CD62L and CD45RB was determined by FACS (D), gates were set using naïve cell from a B6 mouse (as indicated). The percentages of cells within each quadrant is shown. Finally, expression of CD5 was also measured by FACS and the MFI of CD5 expression is indicated in the top left of the plot (E). All plots are representative of 10 individual mice per group.

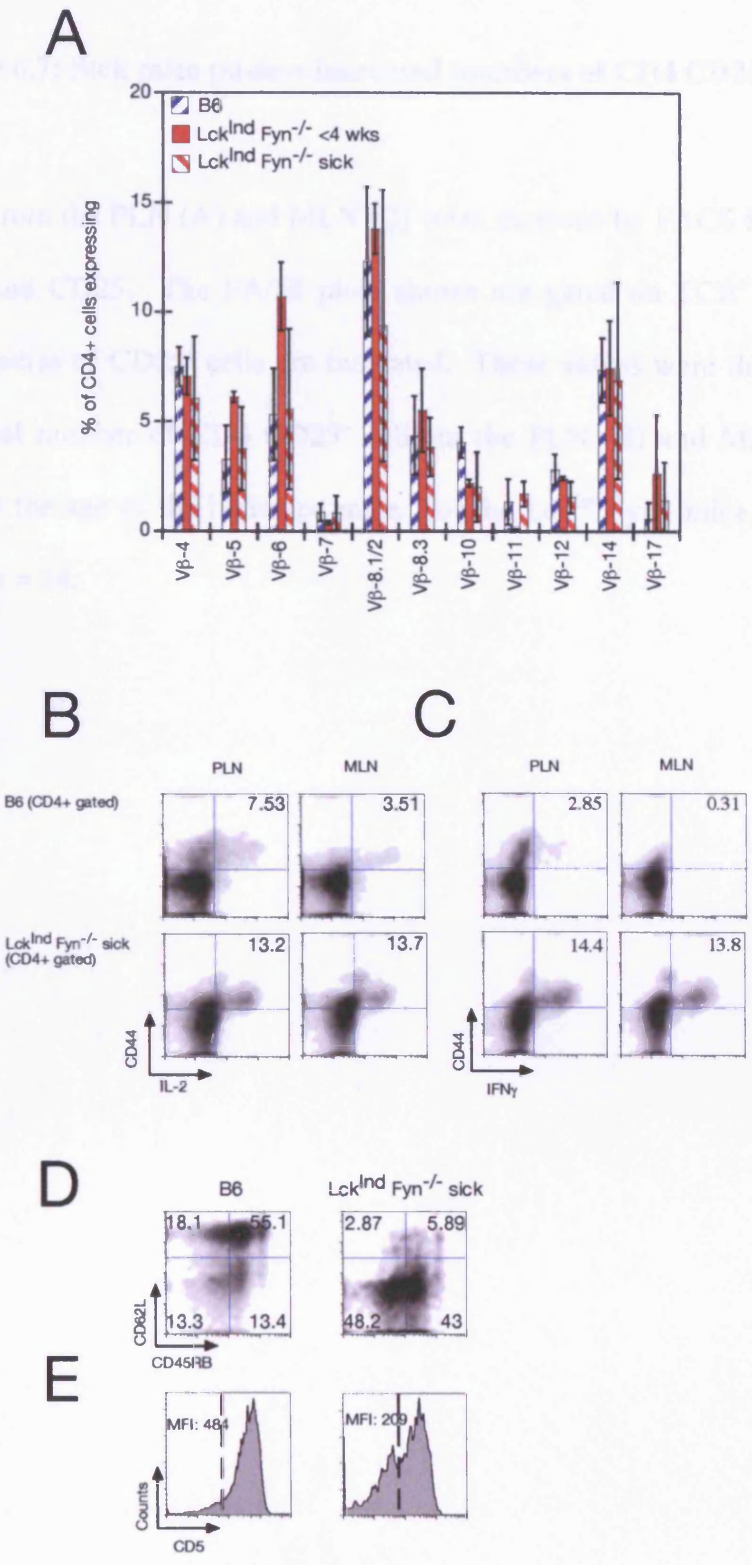

A
B6
Lck^Ind Fyn^-/- <4 wks
Lck^Ind Fyn^-/- sick
% of CD4+ cells expressing
20
15
10
5
0
Vβ-4
Vβ-5
Vβ-6
Vβ-7
Vβ-8.1/2
Vβ-8.3
Vβ-10
Vβ-11
Vβ-12
Vβ-14
Vβ-17
B
C
PLN
MLN
PLN
MLN
B6 (CD4+ gated)
7.53
3.51
2.85
0.31
Lck^Ind Fyn^-/- sick (CD4+ gated)
13.2
13.7
14.4
13.8
CD44
IL-2
CD44
IFNγ
D
B6
Lck^Ind Fyn^-/- sick
18.1
55.1
2.87
5.89
13.3
13.4
48.2
43
CD62L
CD45RB
E
MFI: 484
MFI: 209
Counts
CD5

Figure 6.7: Sick mice possess increased numbers of CD4 CD25$^+$ cells in the LN

Cells from the PLN (A) and MLN (C) were analysed by FACS for the expression of CD4 and CD25. The FACS plots shown are gated on TCR$^+$ CD4$^+$ cells and the frequencies of CD25$^+$ cells are indicated. These values were then used to calculate the total number of CD4 CD25$^+$ cells in the PLN (B) and MLN (C) and plotted against the age of the indicated mice. For the Lck^{Ind} Fyn$^{-/-}$ mice, n = 24; for the B6 mice, n = 14.

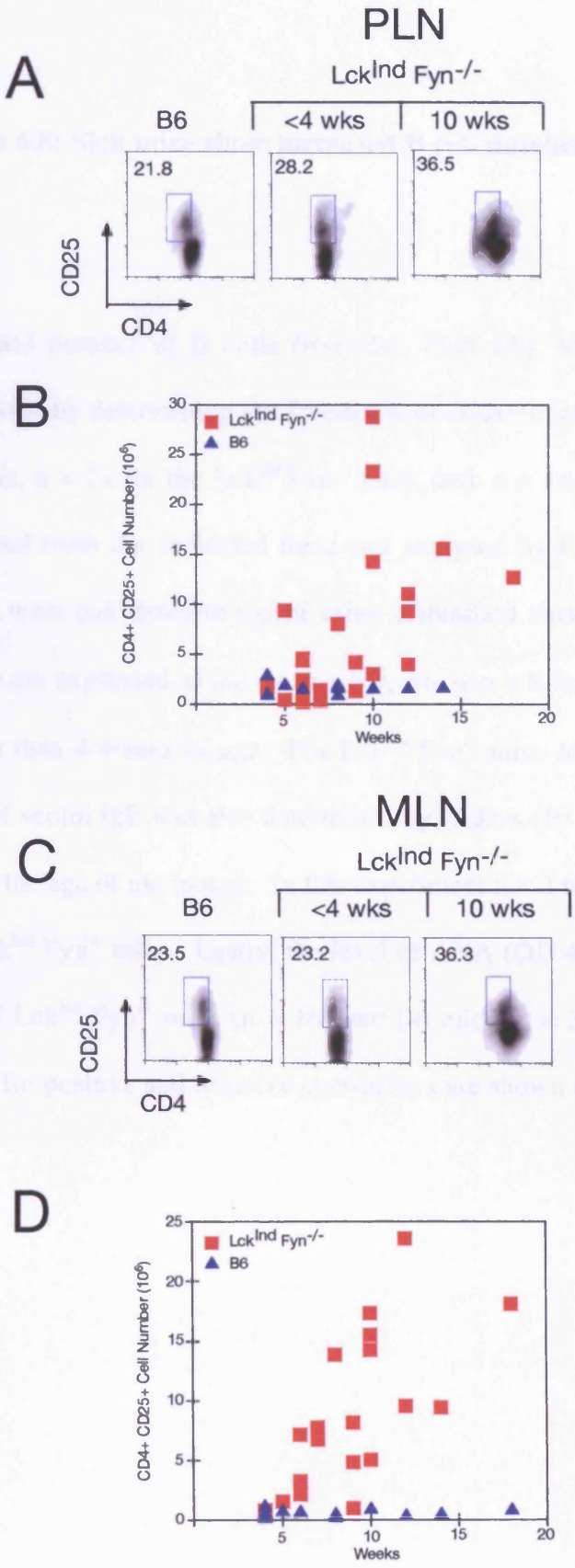

PLN
A
Lck^Ind Fyn^-/-
B6
<4 wks
10 wks
21.8
28.2
36.5
CD25
CD4
B
Lck^Ind Fyn^-/-
B6
CD4+ CD25+ Cell Number (10^6)
Weeks
MLN
C
Lck^Ind Fyn^-/-
B6
<4 wks
10 wks
23.5
23.2
36.3
CD25
CD4
D
Lck^Ind Fyn^-/-
B6
CD4+ CD25+ Cell Number (10^6)
Weeks

Figure 6.8: Sick mice show increased B cell numbers, IgE levels and ANA with age

The total number of B cells from the PLN (A), MLN (B) and spleen (C) was calculated by determining the frequency of $B220^{+}$/CD19+ cells by FACS. For this analysis, n = 24 for the Lck^{Ind} $Fyn^{-/-}$ mice, and n = 14 for the B6 mice. Serum was collected from the indicated mice and analysed by ELISA for Ig levels (D). OD values were converted to μg/ml using a standard curve for each isotype measured. Values are expressed as the mean ± SD, where n = 8 for the B6 and Lck^{Ind} $Fyn^{-/-}$ mice greater than 4 weeks of age. For Lck^{Ind} $Fyn^{-/-}$ mice less than 4 weeks, n = 3. The level of serum IgE was also determined by ELISA (E) and plotted as the OD 450nm versus the age of the mouse. In this experiment n = 3 for the B6 mice, and n = 16 for the Lck^{Ind} $Fyn^{-/-}$ mice. Lastly, the level of ANA (OD 405nm) was determined in the sera of Lck^{Ind} $Fyn^{-/-}$ mice (n = 16) and B6 mice (n = 3) and plotted versus age (F), values for positive and negative control sera are shown on the left of the plot.

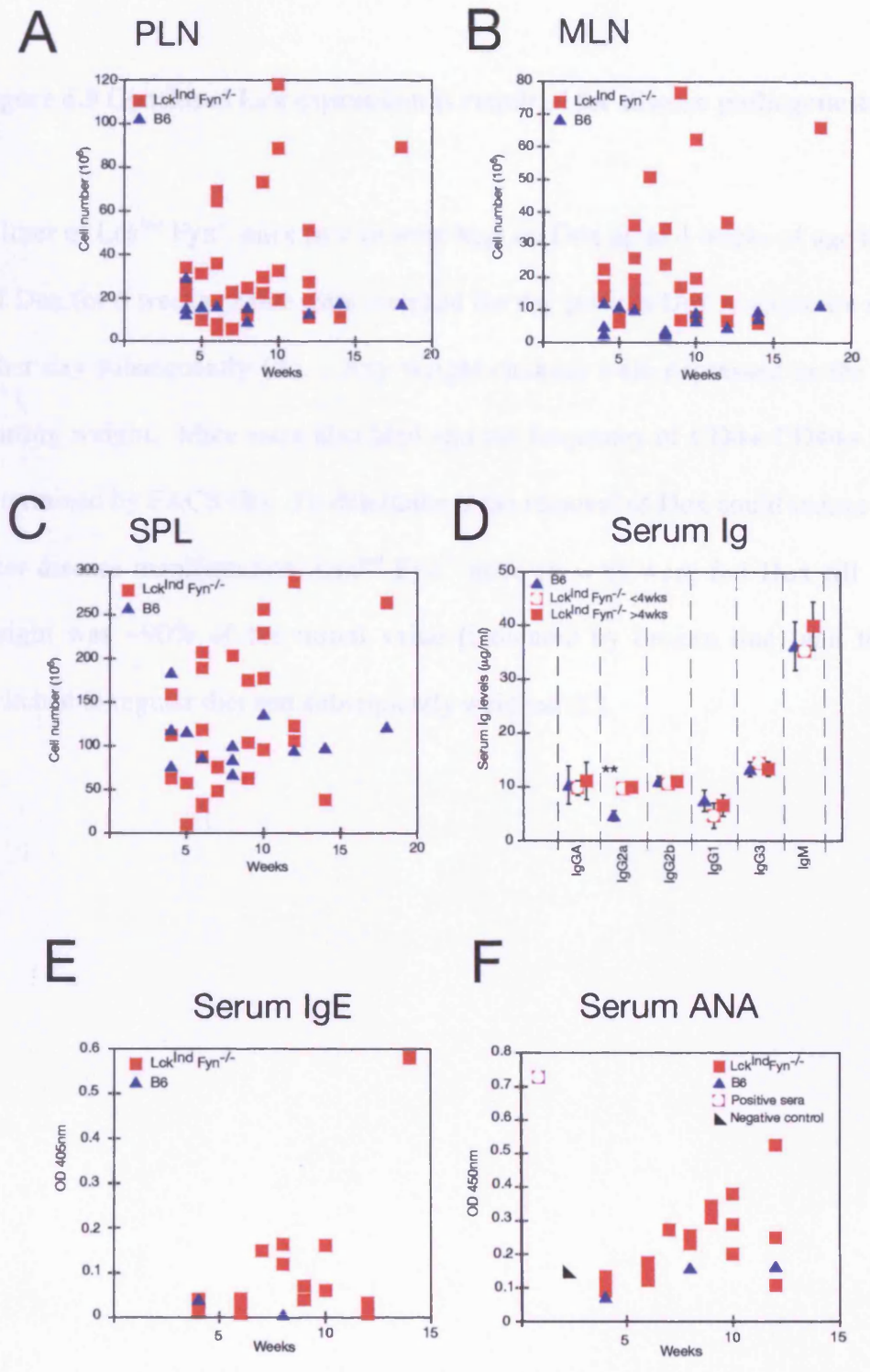
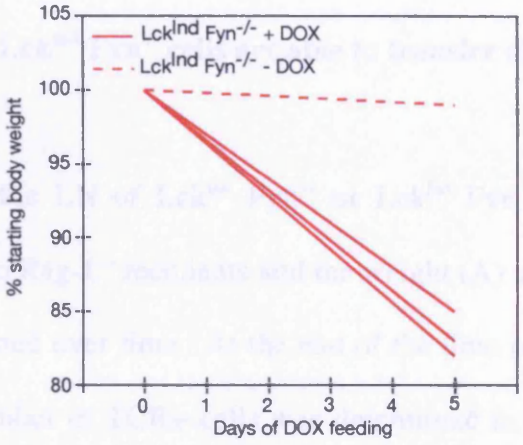

A
PLN
Lck^Ind Fyn^-/-
B6
Cell number (10^6)
Weeks
B
MLN
Lck^Ind Fyn^-/-
B6
Cell number (10^6)
Weeks
C
SPL
Lck^Ind Fyn^-/-
B6
Cell number (10^6)
Weeks
D
Serum Ig
B6
Lck^Ind Fyn^-/- <4wks
Lck^Ind Fyn^-/- >4wks
Serum Ig levels (µg/ml)
**
IgGA
IgG2a
IgG2b
IgG1
IgG3
IgM
E
Serum IgE
Lck^Ind Fyn^-/-
B6
OD 405nm
Weeks
F
Serum ANA
Lck^Ind Fyn^-/-
B6
Positive sera
Negative control
OD 450nm
Weeks

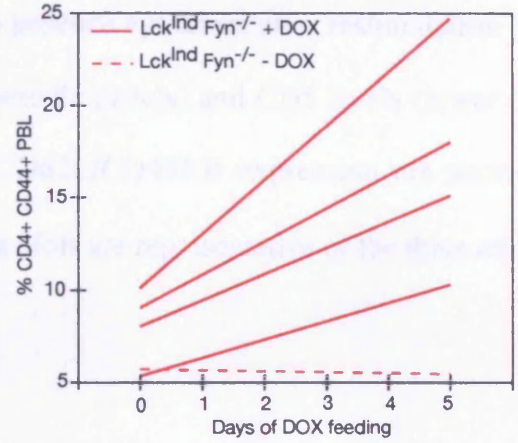
Figure 6.9 Continual Lck expression is required for disease pathogenesis

A litter of Lck^{Ind} $Fyn^{-/-}$ mice (n = 6) were kept on Dox up to 4 weeks of age then taken off Dox for 6 weeks. Mice were weighed the day prior to Dox re-exposure and every other day subsequently (A). Any weight changes were expressed as the % of the starting weight. Mice were also bled and the frequency of CD4+ CD44+ PBL was determined by FACS (B). To determine if the removal of Dox could induce recovery after disease manifestation, Lck^{Ind} $Fyn^{-/-}$ mice (n = 6) were fed Dox till the body weight was ~90% of the initial value (indicated by broken line) and then were switched to regular diet and subsequently weighed (C).

A


Lck^Ind Fyn^-/- + DOX
Lck^Ind Fyn^-/- - DOX
% starting body weight
105
100
95
90
85
80
0
1
2
3
4
5
Days of DOX feeding

B


Lck^Ind Fyn^-/- + DOX
Lck^Ind Fyn^-/- - DOX
% CD4+ CD44+ PBL
25
20
15
10
5
0
1
2
3
4
5
Days of DOX feeding

C

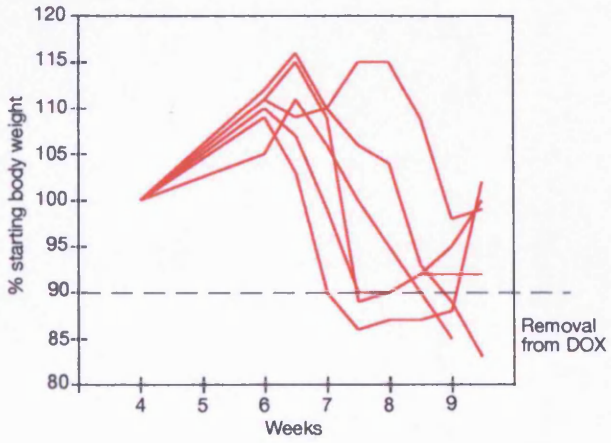

% starting body weight
120
115
110
105
100
95
90
85
80
4
5
6
7
8
9
Weeks
Removal from DOX

Figure 6.10 Lck^{Ind} $Fyn^{-/-}$ cells are able to transfer disease to Rag-$1^{-/-}$ hosts

Cells from the LN of Lck^{Ind} $Fyn^{-/-}$ or Lck^{Ind} Fyn WT mice (as indicated) were transferred to Rag-$1^{-/-}$ recipients and the weight (A) and frequency of CD4+ PBL (B) was determined over time. At the end of the time course, mice were sacrificed and the total number of TCR+ cells was determined in the PLN, MLN and spleen (C) using FACS analysis. These cells were then phenotyped using FACS analysis (D) for the ability to produce cytokines after restimulation (upper panels), CD45RB/CD62L expression (middle panels) and CD5 levels (lower panels). The frequencies for IL-2/IFNγand CD62L/CD45RB expression are shown, as well as the MFI of CD5 levels. These plots are representative of the three animals per treatment group.

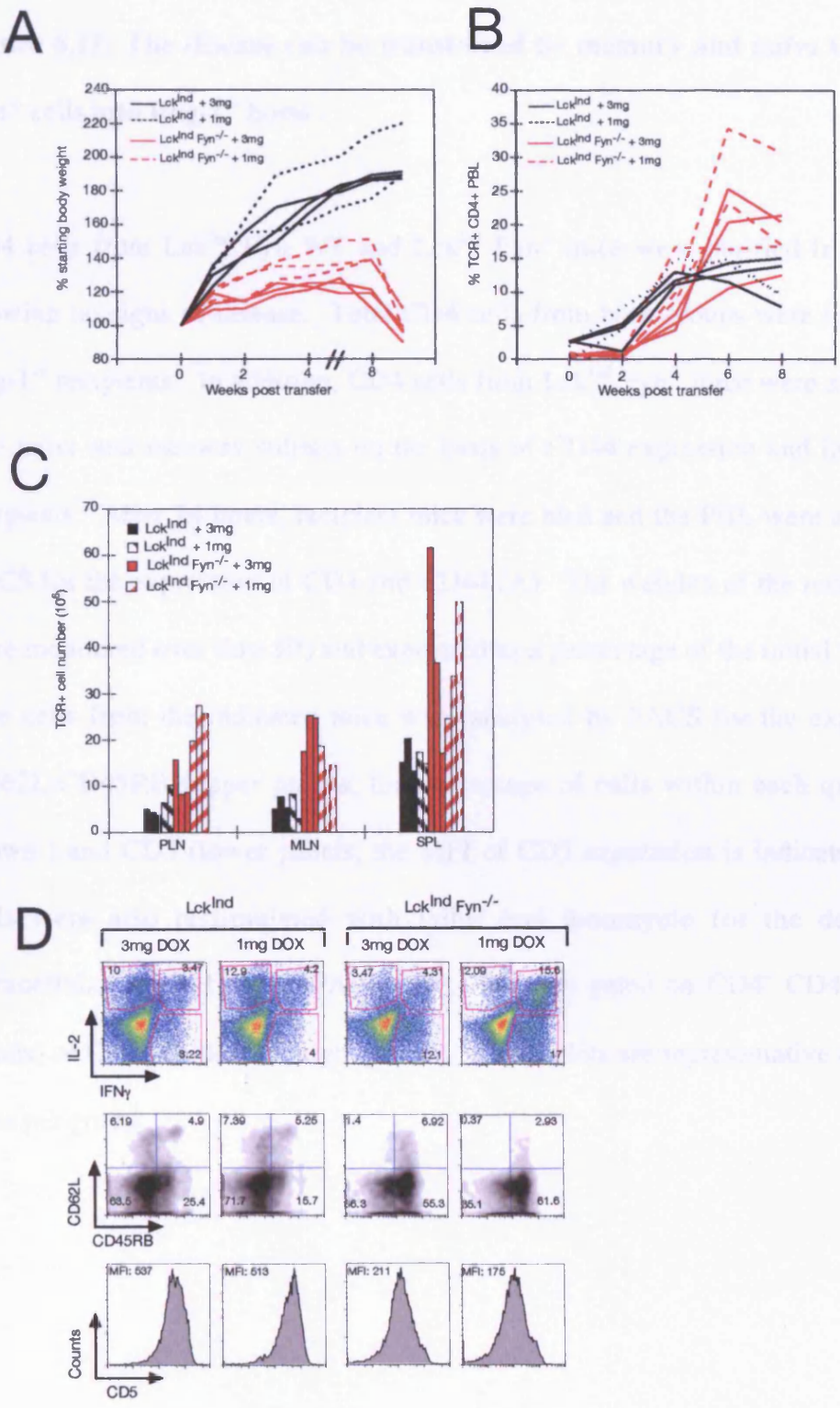

A
B
C
D
% starting body weight
Weeks post transfer
% TCR+ CD4+ PBL
TCR+ cell number (10^6)
PLN
MLN
SPL
Lck^Ind + 3mg
Lck^Ind + 1mg
Lck^Ind Fyn^-/- + 3mg
Lck^Ind Fyn^-/- + 1mg
Lck^Ind
Lck^Ind Fyn^-/-
3mg DOX
1mg DOX
IL-2
IFNγ
CD62L
CD45RB
Counts
CD5
MFI: 537
MFI: 513
MFI: 211
MFI: 175

Figure 6.11: The disease can be transferred by memory and naïve CD4 Lck^{Ind} $Fyn^{-/-}$ cells into Rag-$1^{-/-}$ hosts.

CD4 cells from Lck^{Ind} Fyn WT and Lck^{Ind} $Fyn^{-/-}$ mice were purified from animals showing no signs of disease. Total CD4 cells from both groups were injected into Rag-$1^{-/-}$ recipients. In addition, CD4 cells from Lck^{Ind} $Fyn^{-/-}$ mice were also divided into naïve and memory subsets on the basis of CD44 expression and injected into recipients. After 24 hours, recipient mice were bled and the PBL were analysed by FACS for the expression of CD4 and CD44 (A). The weights of the recipient mice were monitored over time (B) and expressed as a percentage of the initial weight. *Ex vivo* cells from the indicated mice were analysed by FACS for the expression of CD62L/CD45RB (upper panels, the percentage of cells within each quadrant are shown) and CD5 (lower panels, the MFI of CD5 expression is indicated). These cells were also restimulated with PdbU and ionomycin for the detection of intracellular IL-2/IFNγ by FACS (D). Plot are gated on $CD4^+$ $CD45RB^{lo}$ (left panels) or $CD4^+$ $CD45RB^{hi}$ (right panels). These plots are representative of the three mice per group.

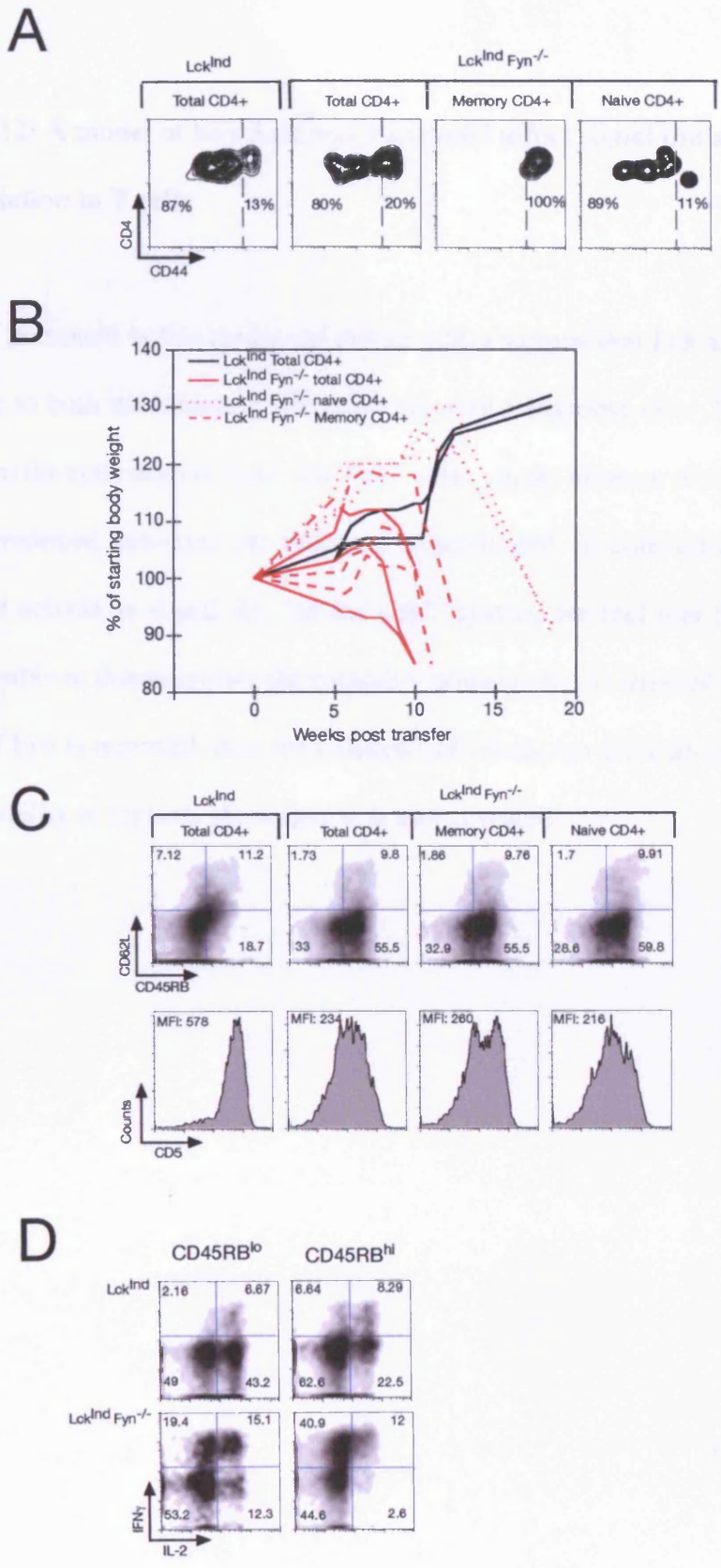
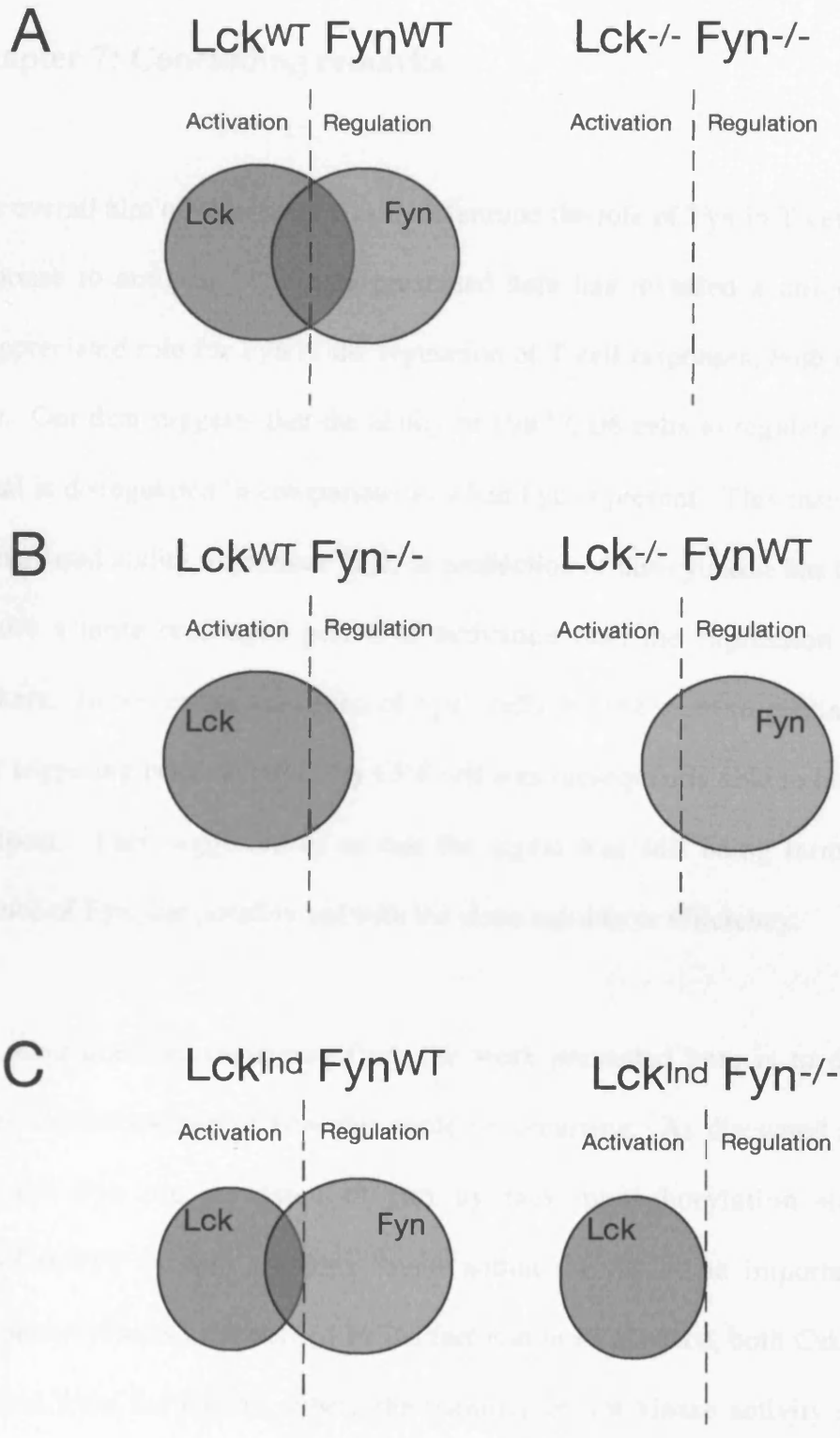

A
Lck^Ind
Lck^Ind Fyn^-/-
Total CD4+
Total CD4+
Memory CD4+
Naive CD4+
87%
13%
80%
20%
100%
89%
11%
CD4
CD44
B
Lck^Ind Total CD4+
Lck^Ind Fyn^-/- total CD4+
Lck^Ind Fyn^-/- naive CD4+
Lck^Ind Fyn^-/- Memory CD4+
% of starting body weight
140
130
120
110
100
90
80
0
5
10
15
20
Weeks post transfer
C
Lck^Ind
Lck^Ind Fyn^-/-
Total CD4+
Total CD4+
Memory CD4+
Naive CD4+
7.12
11.2
18.7
1.73
9.8
33
55.5
1.86
9.76
32.9
55.5
1.7
9.91
28.6
59.8
CD62L
CD45RB
MFI: 578
MFI: 234
MFI: 260
MFI: 216
Counts
CD5
D
CD45RB^lo
CD45RB^hi
Lck^Ind
2.16
6.67
49
43.2
6.64
8.29
62.6
22.5
Lck^Ind Fyn^-/-
19.4
15.1
53.2
12.3
40.9
12
44.6
2.6
IFNγ
IL-2

Figure 6.12: A model of how Lck and Fyn could affect signal initiation and regulation in T cells

The work presented in this thesis and that of others suggest that Lck and Fyn can both contribute to both the initiation and termination of a response (A). This observation stems from the activation of $Lck^{-/-}$ and $Fyn^{-/-}$ cells. In the absence of Fyn, activation is not compromised, however the response is prolonged, in contrast the loss of Lck affects the activation signal (B). In the Lck^{Ind} system, we feel that the reduced Lck level is unable to downregulate the response, however this is masked by the presence of Fyn. If Fyn is removed, then the reduced Lck levels can drive an activation signal, but the inability to regulate the response is now revealed.


A
Lck^WT Fyn^WT
Activation
Regulation
Lck
Fyn
Lck-/- Fyn-/-
Activation
Regulation
B
Lck^WT Fyn-/-
Activation
Regulation
Lck
Lck-/- Fyn^WT
Activation
Regulation
Fyn
C
Lck^Ind Fyn^WT
Activation
Regulation
Lck
Fyn
Lck^Ind Fyn-/-
Activation
Regulation
Lck

Chapter 7: Concluding remarks

The overall aim of this thesis was to determine the role of Fyn in T cell activation in response to antigen. The data presented here has revealed a unique and as yet unappreciated role for Fyn in the regulation of T cell responses, both *in vitro* and *in vivo*. Our data suggests that the ability of $Fyn^{-/-}$ CD8 cells to regulate the activation signal is disregulated in comparison to when Fyn is present. This manifests itself as an increased ability to produce IL-2, as production of this cytokine has been shown to require a more prolonged period of activation than the expression of activation markers. However the activation of $Fyn^{-/-}$ cells did not seem to continue *ad finitum* after triggering because not every CD8 cell was subsequently able to become an IL-2 producer. This suggested to us that the signal was still being terminated in the absence of Fyn, but possibly not with the same rapidity or efficiency.

The main question remaining from the work presented here is to determine the molecular mechanism of how this could be occurring. As discussed in Chapter 3, Lck and Fyn are regulated in part by their phosphorylation status via the PAG/Csk/PEP protein complex found within GEMS. The importance of PAG phosphorylation is underscored by the fact that in its absence, both Csk and PEP are released from the GEMS, where the majority of Src kinase activity is also found (Torgersen *et al.*, 2001). Yasuda et al showed that while $Fyn^{-/-}$ cells had defective PAG Tyr317 phosphorylation compared to WT cells, it was still detectable (Yasuda *et al.*, 2002). This suggests that another kinase is capable of carrying out this action,

but with less efficiency than Fyn itself. Lck has also been shown to phosphorylate PAG using *in vitro* kinase assays, whereas Syk and ZAP-70 could not (Brdicka *et al.*, 2000). Considering all these data, it is possible that the reason for the increase in IL-2 production in the absence of Fyn is due to an increase in the period of Lck activation leading to a more prolonged period of signalling. In this model (Figure 4.12), activation of $Fyn^{-/-}$ T cells would lead to the loss of the weak Tyr 317 phosphorylation of PAG, allowing Lck to become activated and transduce a stimulatory signal. However, without Fyn present, Lck activity would continue for an expanded window of time due to the inefficient rephosphorylation and assembly of the PAG/Csk/PEP complex, possibly by Lck itself. Indeed work by Fillip et al has suggested that the majority of Lck is found outside the GEM in a resting T cell but that Fyn is found within theses lipid domains. This presumably acts to maintain PAG Tyr 317 phosphorylation and keep the T cell inactive, as it is possible to detect residual Fyn kinase activity in resting T cells (Filipp *et al.*, 2003; Yasuda *et al.*, 2002; Haughn *et al.*, 1998). Upon stimulation Lck rapidly translocates into GEMS and may be responsible directly or indirectly for increasing Fyn activity (Filipp *et al.*, 2004; Filipp *et al.*, 2003). This is interesting as it supports the idea that Fyn may act as part of a negative feedback loop to control Lck activity, that is only required when and if Lck is also activated. It is possible that in the absence of Fyn, Lck can phosphorylate Tyr 317 of PAG, but less efficiently than Fyn, as it has to translocate to the GEM in order to encounter PAG. In contrast Fyn is already present there regardless of the T cell activation status. It is also possible that the movement of Lck in an out of the GEM is an extremely dynamic process, explaining why PAG Tyr 317

phosphorylation is reduced, as Lck may only access PAG intermittently. Furthermore, this reduction in efficiency may also be due to the fact that the SH3 domain of Fyn seems to preferentially associate with a proline rich region of PAG (Marie-Cardine *et al.*, 1999), and may reflect differences in substrate specificity between the SH3 domains of Lck and Fyn. Certainly, the SH3 domain of Fyn has been shown to be able to recognise atypical motifs in target proteins including SAP (Latour *et al.*, 2003) and SKAP55 (Kang *et al.*, 2000). It would be interesting to determine if Lck can interact with PAG in the absence of Fyn, and whether the interaction is indeed less efficient.

This model of defective PAG phosphorylation could also offer an explanation for the lymphoproliferative phenotype observed in mice that express no Fyn and reduced Lck (Chapter 6). The reduced level of Lck, while clearly sufficient to transduce activation signals, may not be able to mediate the rephosphorylation of PAG Tyr 317. In a WT mouse, not all Lck is co-receptor associated (Bonnard *et al.*, 1997), however the functional roles of co-receptor and free pools of Lck have not been elucidated. Work form our lab has shown that the Lck protein expressed in the Lck^{Ind} mice is CD4 associated (Lovatt et al., manuscript submitted) and this may mean that the non-co-receptor pool is reduced. It is possible that the absence of non-coreceptor associated Lck in the Lck^{Ind} $Fyn^{-/-}$ mice may contribute to the inability of Lck to effectively substitute for the loss of Fyn in downmodulating a response. The free Lck may be more able to associate with PAG and phosphorylate Tyr 317, mediating assembly of the PAG/Csk/PEP complex. The remaining Lck in the Lck^{Ind} $Fyn^{-/-}$ T

cells could subsequently be disregulated and not subject to the same stringent regulation. This could lead to the conversion of a survival signal delivered by self-pMHC interactions into a proliferative one. In this model, one would expect to be unable to detect any PAG Tyr 317 phosphorylation in a resting naive T cell from the Lck^{Ind} $Fyn^{-/-}$ mice. Furthermore, one may expect that the small amount of Lck present would be in a state of disregulation, as judged by a lack of Tyr 505 phosphorylation and an increase in Tyr 394 phosphorylation. We plan to look at this in future experiments.

It is unlikely that the putative absence of PAG Tyr 317 phosphorylation in the sick mice is solely responsible for this phenotype because $PAG^{-/-}$ mice are reported to remain healthy (Dr J Lindquist, personal communication). However, PAG also contains 10 Tyr residues that can be phosphorylated, 6 of which are arranged into an atypical ITAM motif (Lindquist *et al.*, 2003). Therefore it is possible that some residues may mediate different functions, some positive and some negative with respect to T cell activation. Interestingly, when we IP PAG from CD4 cells of the sick Lck^{Ind} $Fyn^{-/-}$ mice and probe for total phosphotyrosine using the 4G10 mAb, we observed an increase in the total phosphorylation of PAG compared to controls that include Lck^{Ind}, B6 and $Fyn^{-/-}$ CD4 cells (data not shown). However, there were more $CD44^{hi}$ cells in the CD4 population isolated from Lck^{Ind} $Fyn^{-/-}$ mice and this may have contributed to the differences observed. It is possible that there is a defect in the phosphorylation of Tyr 317 that would be masked by an increase in the signal from other Tyr residues, and that the increase in phosphorylation of other Tyr residues may

play some inductive role in the T cell lymphoproliferation explaining why $PAG^{-/-}$ mice remain healthy. We plan to look at this directly using a pTyr 317-specific antibody.

Another explanation as to why $PAG^{-/-}$ mice remain healthy is the fact that the adapter molecule LIME has also been shown to bind Csk/PEP complexes (Brdickova *et al.*, 2003). Furthermore, it is thought that LIME phosphorylation is mediated by Lck, and in the absence of PAG, LIME may also be able to also regulate Src kinase signalling. Interestingly, when LIME is phosphorylated, the SH2 domain of Lck can bind to it. The result of this interaction is that even though Csk can phosphorylate Tyr505 on Lck, the kinase domain remains open as the SH2 domain is bound to phospho-LIME. Therefore, LIME may only be able to allow PEP to dephosphorylate the kinase promoting Tyr394 of Lck, but prevents molecular closure from occurring. The net result of this may be some residual Lck kinase activity. From these data, one could hypothesis that the ability of LIME/Csk/PEP complex to regulate a signal may not be as effective as the PAG/Csk/PEP complex. Considering these data it may be of interest to look at the role of LIME in both the F5 CD8 $Fyn^{-/-}$ and CD4 cells from the Lck^{Ind} $Fyn^{-/-}$ systems. This model is further complicated by the fact that LAT also binds Csk/PEP complexes and also Lck within the GEMs (Kabouridis, 2003).

The data presented in this thesis shows that Fyn deficiency has two very different outcomes depending on the level of Lck also present. In one situation where we have no Fyn but WT Lck levels, it could be argued that Fyn deficiency is beneficial to the

generation of a CTL response by virtue of the increase in autocrine IL-2 production (Chapter 5). This is an interesting observation as IL-2 therapy has been used as a treatment for cancer to improve the activity of CTL responses against tumour cells in mouse models and human clinical trials (Radny *et al.*, 2003; Shrikant and Mescher, 2002; Rosenberg *et al.*, 1985). The success has been variable, as large does administration of recombinant IL-2 has led to life threatening disorders in patients such as capillary leakage syndrome (Rosenstein *et al.*, 1986). It is thought that this may be due to the fact that the administration of IL-2 is far from localised and will not only influence CD8 cells. In terms of the use of mouse models, situations where the tumour has been engineered to secrete localised IL-2 has led to improved CTL activity and clearance of the tumour (Schweighoffer *et al.*, 1996; Boyer *et al.*, 1995). Furthermore, studies have attempted to transfect the tumour infiltrating T cells themselves with an IL-2 transgene to overcome localisation problems (Liu and Rosenberg, 2003). If we could look in future at the ability of F5 $Fyn^{-/-}$ mice to deal with persistent antigens such as tumour cells expressing the NP68 peptide, we may expect that in the absence of Fyn, survival and control of tumour growth may be improved compared to Fyn sufficient controls.

IL-2 therapy has also been used in clinical trial to treat HIV infection in conjunction with HAART (Martinez-Marino *et al.*, 2004a; Martinez-Marino *et al.*, 2004b; Kovacs *et al.*, 2001; Kovacs *et al.*, 1996). With HIV infection, although the immune system is considered to be in a perpetual state of chronic activation, CTL function may be impaired as HIV specific CD8 cells possess very little Perforin (Andersson *et al.*,

1999) and cytolytic activity (Appay *et al.*, 2000; Shankar *et al.*, 2000; Gray *et al.*, 1999; Trimble and Lieberman, 1998). As discussed in Chapter 5, the production of IL-2 by CD8 cells and the subsequent expression of Perforin could be the limiting factor in terms of the duration and effectiveness of a CTL response. While autocrine IL-2 consumption is able to generate CTL, sustained responses against persistent infections such as HIV may require CD4 help in the form either paracrine IL-2 or indirectly via CD40-CD40L interactions (Schoenberger *et al.*, 1998). In HIV patients, CD4 numbers decline over time, therefore the help that sustained CTL function would need may also be diminished. As our data has shown in the F5 system that in the absence of CD4 cells, IL-2 plays a significant part in the generation/function of CTL. Therefore, the administration of exogenous IL-2 is thought to act to sustain CTL function by regulating Perforin expression in HIV treatment.

Interestingly, elevated Fyn activity has been reported in CD8 T cells from HIV infected individuals (Phipps *et al.*, 1997; Phipps *et al.*, 1996). Although primarily thought to infect CD4 cells, there is accumulating evidence that HIV can also infect CD8 cells (Saha *et al.*, 2001; Flamand *et al.*, 1998; Yang *et al.*, 1998; Livingstone *et al.*, 1996; Mercure *et al.*, 1993), furthermore the NEF protein of HIV can interact with the SH3 domain of Src kinases (Collette *et al.*, 1996), including Fyn (Arold *et al.*, 1997). There could be functional significance to this interaction because, as well as the intramolecular association of the C-termini Tyr residue with the SH2 domain, it has also been shown that the SH2-CD linker region between the SH2 and kinase

domains can associate with the SH3 domain and stabilise the closed molecular conformation that interferes with kinase activity (Gonfloni *et al.*, 1997). While the mechanisms controlling this are unclear (Briggs *et al.*, 2000), it is interesting that the NEF protein has been shown to displace this interaction *in vitro,* due to the presence of a PxxP motif (Moarefi *et al.*, 1997). Therefore binding of the NEF protein to the SH3 domain of Fyn may reflect a mechanism by which the virus elevates Fyn activity by preventing the SH3 domain from interfering with catalytic site. Certainly, the recently described activator of Lck and Fyn, Unc199, has been shown to act in a similar manner (Gorska *et al.*, 2004). In terms of our findings, this would presumably reduce IL-2 production and affect CD4 independent CTL function. Therefore the combination of putative increased Fyn kinase activity coupled with the loss of CD4 cell numbers may contribute to a failure to generate long lasting CTL activity, making the ability to boost IL-2 production by CD8 cells especially relevant in CD4 depleted environments. However, it should also be noted that the role of Src kinases in HIV pathogenesis still remains to be fully elucidated as there are also reports suggesting that the activity of these molecules are in fact reduced in HIV infected individuals (Guntermann *et al.*, 1997; Morio *et al.*, 1997).

Considering that an increase in CTL activity could be beneficial in HIV and cancer treatments, it may be of interest to consider situations where Fyn activity could be inhibited by a pharmacological reagent. This could possibly both increase and prolong a CTL response by elevating IL-2 production, mimicking the effect of IL-2 therapy. However, it would have certain advantages over the administration of IL-2

to patients. For example as the increase in IL-2 production would in theory be CD8 cell specific and highly localised, therefore it may overcome any potential toxicity problems resulting from high doses of IL-2. Furthermore, there are studies that suggest that the period of IL-2 administration during an immune response is critical as it may even have a detrimental effect on memory T cell survival (Dai *et al.*, 2000; Ku *et al.*, 2000), and may be most beneficial during the contraction phase of a response (Blattman *et al.*, 2003). Inhibition of Fyn in CD8 cells may overcome this problem as the window of IL-2 production would not be grossly different from Fyn sufficient cells and thus the period of IL-2 exposure for the CD8 cells should not be changed.

As discussed in Chapters 3 and 4, Fyn may regulate the induction of anergy in CD8 T cells (Welke and Zavazava, 2002; Utting *et al.*, 2001; Utting *et al.*, 2000; Gajewski *et al.*, 1994). In situations where antigen persists, studies have suggested CD8 cells become anergic (Ehl *et al.*, 1998), and this may contribute to the failure of CTL responses in HIV and cancer. Therefore, selective inhibition of Fyn may be able to break this anergic state and allow CTL function to continue indefinitely; something that would be especially beneficial to HIV treatment. Moreover, the pharmacological targeting of a host cellular protein such as Fyn, as opposed to a viral protein that is subject to high rate mutation may overcome the problems with drug resistance that has become so prevalent in HIV infection management.

There are however a number of caveats to this idea. Firstly, inhibition of Fyn would presumably also affect other cell types that require this kinase to be active. Mast cells also express Fyn, however it seems to be important for transducing degranulation signals (Parravicini *et al.*, 2002), and therefore inhibition may not be detrimental in terms of an anti-viral response. In CD4 cells, Fyn seems to play a role in regulating the production of Th2 cytokines (Cannons *et al.*, 2004; Davidson *et al.*, 2004). Again, in terms of viral clearance there may be a preferential skewing to Th1 type CD4 cells and the loss of Fyn did not seem to affect IFNγ production by these cells after Th1 polarisation (Cannons *et al.*, 2004; Davidson *et al.*, 2004). The role of Fyn in B-cells has not been fully elucidated, however Fyn deficiency does not seem to lead to any overt phenotype (Horikawa *et al.*, 1999). The splice variant FynB is also expressed in the brain, in a form that utilises exon 7A of the Fyn gene leading to alterations in the kinase domains of FynT and FynB (Davidson *et al.*, 1994). FynB deficient mice have been shown to have defects in the myelination of oligodendricytes (Sperber *et al.*, 2001; Osterhout *et al.*, 1999; Umemori *et al.*, 1999) and also exhibit a number of behavioural defects such as increased fear responses (Miyakawa *et al.*, 1994). While it could, in theory, be possible to develop an inhibitor that would only exert an effect of FynT, the blood/brain barrier may prevent any compound from being able to target FynB. However, one major obstacle to the use of Fyn inhibitors to boost CTL function may be the need for specific cell type delivery to CD8 cells.

In any case, it may be beneficial to use a Fyn inhibitor for short periods. For example, current IL-2 therapy for the treatment of HIV involves the administration of this cytokine during an STI (structured antiviral therapy interruption) of HAART (Smith *et al.*, 2000). In this case, viral replication is no longer pharmacologically suppressed and viral loads begin to peak again. However, the magnitude of this peak was inversely proportional to the CTL response mounted by the patient during the STI. Therefore IL-2 treatment during this period is thought to boost CTL function eventually leading to a lower, stabilised viral load. It may be possible to use an inhibitor of Fyn in the same manner and assess the impact on CTL function by virtue of the steady state viral load achieved. However, there are suggestions from our data, that while primary effector function may be increased, memory CD8 generation could be compromised in our system in the absence of Fyn. This would also need to be considered in terms of disease management.

The major caveat to the use of pharmacological Fyn inhibitors would be the degree of Src kinase specificity. While Fyn deficiency could be argued as beneficial to CTL responses, if Lck expression / activity was also reduced by the inhibitor we may then generate a situation resembling the Lck^{Ind} $Fyn^{-/-}$ mouse disease model. Therefore, any Fyn inhibitor would have to have a high degree of specificity and not interfere with the actions of Lck. There are structural differences between Fyn and Lck that could be exploited to produce a compound that may inhibit action of one and not the other. For example the primary sequence of the SH2-CD linker places these two kinases into two different phylogenetic groups (Williams *et al.*, 1998). Furthermore this

linker region may mediate the specific interaction of Fyn with the molecule SAM68 (Feuillet *et al.*, 2002). If the binding of NEF to this region is responsible for increasing Fyn kinase activity and rendering CD8 cells anergic, then designing a small molecule inhibitor that could prevent NEF binding to the SH-2-CD linker of Fyn without affecting molecular conformation may be a possible strategy. Alternatively, it may also be possible to target Fyn specifically by virtue of the unique region, as this would presumably not affect Lck function, as there is no sequence similarity within these domains.

There are two possible ways to look at the use of Fyn inhibitors as a way of boosting an immune response. As mentioned previously, in terms of a response against a virus or cancer cell it may prove beneficial to boost a CTL response. However, in situations of autoimmunity where the focus of the immune system is turned toward self, the loss of Fyn may prove detrimental to the host. Certainly, this was the case when we introduced Fyn deficiency into mice that expressed reduced Lck levels. We are currently planning to look at the role of Fyn in a number of experimental autoimmune models to see if a deficiency in this kinase will exacerbate the disease.

Considering the potential positive and negative effects that Fyn could have on an immune response it will be essential to determine the downstream targets of Fyn, and furthermore, what the contributions of the SH2, SH3, SH4 and kinase domains are to these processes.

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